REMARKS

Claims 1, 4-8, 10, 14, 15, 17, 18, 20, and 22-26 are pending. Claims 1, 4-8, 20, 22, 23, 25, and 26 are rejected under 35 U.S.C. § 112, first paragraph. Claims 1, 4-8, 10, 14, 15, 18, 20, and 22-26 are rejected under 35 U.S.C. § 112, second paragraph. Claims 10, 14, 15, 18, 20, and 24 are rejected under 35 U.S.C. § 103. Claims 10, 14, 15, 18, 20, and 24 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting. Applicants address each of these bases for rejection as follows.

As an initial matter, Applicants wish to thank Examiners Zachary Howard and Ruixiang Li for the helpful interview conducted with Applicants' representative James DeCamp on November 14, 2006.

Claim amendments

Claim 1 has been amended to recite a proliferation inducing part of c-mpl. Support for this amendment is found throughout the specification. For example, at page 5, lines 1-4, the specification states:

> [T]he "domain comprising a cytokine receptor or a part thereof that imparts proliferation activity to a cell upon the association" is derived from a G-CSF receptor or a c-mpl.

In addition, Example 8 provides exemplary fusion proteins containing a c-mpl cytoplasmic domain which, when expressed by a cell, result in ligand-inducible cell proliferation (see e.g., Figure 23).

Claim 10 has been amended to delete the word "a" in reference to c-mpl. No new matter has been added by the present amendments.

Applicants reserve the right to pursue any canceled subject matter in this or in a continuing application.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 1, 4-8, 20, 22, 23, 25, and 26 are rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement in containing new matter. In particular, the Office asserts (page 4):

> The Examiner cannot find any teaching in the specification describing a receptor comprising the full-length G-CSFR extracellular domain and the cytoplasmic domain of c-mpl. Therefore, there is no conception of this specific genus of molecules in the specification, nor does the concept of the specific genus flow naturally from the disclosure.

Applicants respectfully disagree.

The specification, at page 4, lines 13-16, states:

> The present invention relates to a fusion protein comprising a ligand-binding domain, a domain that associates when a ligand binds to the ligand binding domain, and a domain that imparts proliferation activity to a cell upon association.

In addition, the specification, at page 7, lines 18-22, states:

> As the "domain which imparts proliferation activity to a cell" of the fusion protein according to the present invention, it is possible to use a molecule that transmits the intracellular proliferation signal, for example, an entire molecule of a cytokine receptor. It is also possible to use only a domain in a molecule that imparts proliferating activity to a cell.

And, at page 5, lines 1-4, the specification teaches that "the 'domain comprising a cytokine receptor or part thereof that imparts proliferation activity to a cell upon the association' [may be] derived from a G-CSF receptor or a c-mpl."

Clearly the invention described in the specification encompasses various combinations between domains of cytokine receptors (including the G-CSF receptor and the Thrombopoietin receptor c-Mpl). Moreover, the specification provides numerous examples of fusion proteins containing domains from two different receptors. For instance, in Figure 14D shows a fusion protein containing *the full-length extracellular domain of the G-CSF receptor* and a mutant estrogen receptor specific for 4-hydroxytamoxifen (TmR). Also, in Figure 14, the specification provides a schematic diagram of the G-CSF Receptor with *the full-length extracellular domain of the G-CSF receptor* clearly labeled (see Figure 14A). Further, for instance, in Example 8, the specification describes the construction of fusion proteins containing a deleted G-CSF receptor extracellular domain and *the c-mpl cytoplasmic domain.*

In view of the various examples in Applicants' specification of fusion proteins containing the full-length G-CSF receptor extracellular portion and fusion proteins containing the c-mpl cytoplasmic portion, Applicants submit that one skilled in the art would recognize that other combinations of the disclosed domains, including a fusion protein containing the full-length G-CSF receptor extracellular portion and the c-mpl cytoplasmic portion are within the scope of the invention described in the specification as

filed. This basis for the written description rejection may be withdrawn.

The Office also asserts (page 4):

> [C]laim 25 is directed to a kit comprising two vectors (the vector of claim 7 or claim 10 and another vector comprising an exogenous gene) and a ligand. The Examiner cannot find any teachings in the specification describing a kit with two vectors and a ligand. (Emphasis original.)

In response, Applicants note that the specification, at page 7, line 26, to page 8, line 5, teaches:

> *[T]he vector used in the present invention includes* not only a single vector molecule containing the fusion protein-coding DNA and a single vector molecule containing the fusion protein-coding DNA and the exogenous gene, but also includes *a vector system of multiple vector molecules comprising a combination of a vector containing the fusion protein-coding DNA and a vector containing the exogenous gene*, for example, a binary vector system. (Emphasis added.)

In addition, the specification, at page 6, lines 24-26, states:

> A kit comprising (a) the vector of (7) or (10), and (b) a ligand capable of acting on the "ligand-binding domain" of the fusion protein encoded by the gene contained in the vector.

The term "the vector of (10)" is described at page 5, lines 23-24, of the specification as "A vector comprising a desired exogenous gene and a gene encoding a fusion protein." As noted above, at page 7, line 26, to page 8, line 25, the specification defines a "vector" to include a vector system containing a vector including the fusion protein-coding DNA and a vector containing the exogenous gene. Hence, the kit described at page 6, lines 24-26, can include two vectors (one encoding the fusion protein and one containing the exogenous gene) as well as a ligand. This basis for the written description rejection may

also be withdrawn.

In addition, the Interview Summary mailed on December 11, 2006 states that support for the genus of tamoxifen derivatives and metabolites was discussed during the November 14, 2006 interview. Applicants note that the specification states (page 7):

> [A] tamoxifen, the derivative thereof (ex. Tremifen), or the metabolite thereof (ex. 4-hydroxytamoxifen) can be preferably used.

Derivatives of tamoxifen and metabolites of tamoxifen were well known in the art at the time of filing. In this regard, Applicants direct the Office's attention to the enclosed copies of Gulino and Pasqualini (Cancer Research 42:1913-1921, 1982; "Gulino;" Exhibit 1) and Lien et al. (Cancer Research 49:2175-2183, 1989; "Lien;" Exhibit 2). In particular, in the list of chemicals (bottom of right column, page 1913) Gulino lists tamoxifen Metabolites A, B (monohydroxytamoxifen), D (dihydroxytamoxifen), and X (*N*-desmethyltamoxifen) as well as ICI Pharma catalog numbers for these derivatives. In the abstract, Gulino describes Metabolites B and D as hydroxylated derivatives of tamoxifen. Lien, in the abstract, lists additional metabolites of tamoxifen; namely 4-hydroxy-*N*-desmethyl-tamoxifen (Metabolite BX), the primary alcohol (Metabolite Y), and *N*-desdimethyltamoxifen (Metabolite Z). Clearly, at the time of filing, there was an extensive knowledge in the art of tamoxifen derivatives and metabolites.

In sum, in view of the description of exemplary tamoxifen derivatives and metabolites in the specification and the knowledge in the art at the time of filing, Applicants submit that genus of derivatives of tamoxifen and tamoxifen metabolites finds

adequate written description in Applicants' specification as filed.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 1, 4-8, 10, 14, 15, 18, 20, and 22-26 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite. In particular, the Office states (page 5):

> Claim 1 is indefinite because it is unclear what is modified by the phrase "or a proliferation inducing part thereof". It is unclear whether "or a proliferation inducing part thereof" modifies "the cytoplasmic domain of c-mpl", "the extracellular region of a granulocyte colony stimulating factor receptor", or both.

Claim 1 has been amended to recite a proliferation inducing part of the cytoplasmic region of c-mpl. Applicants submit that the amendment to claim 1 overcomes this basis for rejection.

The Office also states that claim 10 is indefinite because it recites "a c-mpl" and that deleting the word "a" would render the claim definite. The word "a" has been deleted in the present amendment to claim 10. This basis for rejection may be withdrawn.

Rejection under 35 U.S.C. § 103

Claims 10, 14, 15, 18, and 20 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Ito et al. (Blood 90:3884-3892, 1997; "Ito") in view of Drachman et al. (Proc. Natl. Acad. Sci. USA 94:2350-2355, 1997; "Drachman"). Claim 24 is rejected under 35 U.S.C. § 103(a) as being unpatentable over Ito in view of Drachman and further

in view of Picard ((Ch 11 pp. 261-274) in Nuclear Receptors: a Practical Approach (D. Picard, ed.) Oxford University Press, Oxford, 1999; "Picard").

Claims 10, 14, 15, 18, and 20

The Office states (page 6):

> It would have been obvious to the person of ordinary skill in the art at the time the invention was made to substitute c-mpl as taught by Drachman for G-CSFR in the vector taught by Ito, and to further include an exogenous gene encoding neomycin resistance as taught by Drachman.

Applicants respectfully disagree.

Drachman describes functional elements of the Mpl cytoplasmic domain as well as vectors containing such functional elements and the Drachman vectors contain a neomycin resistance gene. Drachman fails to describe a vector encoding a *fusion* protein, much less a fusion protein containing the ligand-binding domain of a steroid hormone receptor and c-mpl. Drachman in no way teaches or suggests generating a vector encompassed by claim 10.

As noted in Applicants' last reply, Ito describes vectors containing a DNA encoding a fusion protein including a granulocyte colony stimulating factor receptor and the hormone-binding domain of an estrogen receptor. Ito does not describe c-mpl.

The Office states (page 6):

> The person of ordinary skill in the art would be motivated to [combine the teachings of Ito and Drachman] in order to use the vector to selectively amplify Ba/F3 hematopoietic cells, and because Ito suggests modifications using other growth factor receptor genes such as the erythropoietin receptor.

And, in response to Applicants' argument that Ito, in fact teaches away from the desirability of making a vector expressing a fusion protein with a signal transducing portion other than a portion from G-CSFR, the Office states (page 7):

> Ito teaches other uses for the chimeric receptors than clinical in vivo application. Specifically, Ito teaches that the chimeric receptors can be used for ex vivo amplification of cells.

Applicants submit that the passages referred to by the Office refer to *ex vivo* amplification prior to *in vivo* use. In particular, Ito states (page 3884, left column):

> Although retroviral vectors are widely used to deliver genes into hematopoietic stem cells, the transduction efficiency has been too low to obtain the expression level required for improvement of clinical manifestations. There are several approaches to the problem ... selection and enrichment of transduced hematopoietic stem cells ex vivo may be a feasible approach.

Hence, Ito suggests *ex vivo* expansion of transduced cells to overcome the problems associated with low transduction efficiency. Nonetheless, the cells containing the vector (i.e., the transduced cells) are expanded. The purpose of the expansion is clearly to produce a sufficient number of transduced cells to obtain a cell population with an expression level required for improvement of clinical manifestations. Consequently, because the cells are expanded for *in vivo* use, the statement in Ito that "the safe use of rhG-CSF in humans suggests that the signals from exogenously expressed G-CSFR-derived molecules are safer than those of other receptors" also applies to *ex vivo* amplification.

For the reasons set forth above, Applicants maintain that Ito, while mentioning possible chimeras containing other growth factor receptor (*c-kit* or erythropoietin receptor) portions, clearly teaches away from the desirability of making vector expressing a chimera with a signal transducing portion other than a portion from G-CSFR.

Applicants note that a *prima facie* case of obviousness cannot be maintained where the prior art teaches away from the claimed invention. As the Federal Circuit reiterated in reversing a Board decision finding obviousness:

> A prima facie case of obviousness can be rebutted if the applicant ... can show 'that the art in any material respect taught away' from the claimed invention .... *A reference may be said to teach away when a person of ordinary skill, upon reading the reference, ... would be led in a direction divergent from the path that was taken by the applicant.*

In re Haruna, 249 F.3d 1327, 1335, 58 U.S.P.Q.2d 1517, 1522 (Fed. Cir. 2001) (emphasis added) (citations omitted).

For all the reasons, Applicants submit that the 35 U.S.C. § 103 rejection of claims 10, 14, 15, 18, and 20 should be withdrawn.

Claim 24

Applicants note that claim 24 depends from claim 10. Claim 10 was not included in this basis for rejection and Applicants submit, for the reasons set forth above, that claim 10 is non-obvious over the cited art. As such, Applicants submit that, for the same reasons, dependent claim 24 is also non-obvious. This basis for the § 103 rejection

should also be withdrawn.

Provisional rejection for nonstatutory obviousness-type double patenting

Claims 10, 14, 15, 18, 20, and 24 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting over claims 8, 12, 15, 17, and 22 of co-pending application serial number 09/905,592 ("the '592 application") in view of Ito and Drachman. Applicants agree to address this basis for rejection, if appropriate, upon an indication of otherwise allowable subject matter.

CONCLUSION

Applicants submit that the application is now in condition for allowance, and such action is hereby respectfully requested.

Applicants hereby request an interview to discuss the present reply once the Office has had time to review Applicants' response. As such, Applicants would appreciate it if the Examiner were to contact the undersigned in due time.

Enclosed is a Petition to extend the period for replying to the Office Action for three (3) months, to and including February 23, 2007, and a check in payment of the required extension fee.

If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 23 February 2007

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Heterogeneity of Binding Sites for Tamoxifen and Tamoxifen Derivatives in Estrogen Target and Nontarget Fetal Organs of Guinea Pig[1]

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ABSTRACT

The present paper shows that the antiestrogen tamoxifen and different tamoxifen derivatives bind to two distinct cytoplasmic binding sites in the fetal uterus of guinea pig. The first one (Site A) corresponds to the estrogen receptor and binds tamoxifen with a dissociation constant (K_d) of 1.8 ± 0.4 nM. The dissociation rate constant (k_{-1}) of the Site A:tamoxifen complex at 4° is 8.3 ± 2 × 10^{-4} sec^{-1} and at 26° is 123 ± 26 × 10^{-4} sec^{-1}. The binding ability of Site A appears to be thermolabile, being destroyed by heating at 37°. The hydroxylated derivatives of tamoxifen (Metabolites B and D) have a higher affinity for Site A as compared to tamoxifen. The second binding site for tamoxifen (Site B) appears to be specific for the triphenylethylene class of antiestrogens (nafoxidine and several tamoxifen metabolites), the hydroxylation of tamoxifen decreasing the affinity for Site B. In contrast, natural and synthetic estrogens as well as cortisol, testosterone, and progesterone do not compete for Site B. Site B shows a higher affinity for tamoxifen (K_d 0.39 ± 0.01 nM) as compared to Site A and a higher stability of the complex at both 4 and 26° (k_{-1} 0.81 ± 0.14 and 3.0 ± 0.4 × 10^{-4} sec^{-1}, respectively). The binding ability of Site B appears to be resistant to heating at 37°. Both Sites A and B are destroyed by proteolytic treatment and are precipitated by 36% saturated ammonium sulfate. The tamoxifen:Site A complex translocates into the uterine nuclei in a "cell free" system by a temperature-dependent process, but the tamoxifen:Site B complex does not. Nevertheless, a site with a K_d similar to cytoplasmic Site B (0.47 ± 0.1 nM) is spontaneously present in untreated fetal uterine nuclei. In other fetal organs which contain no estrogen receptor (heart) or very low levels (lung), the concentration of Site B is found to be significantly lower than in the fetal uterus (5 to 6 times). Furthermore, progressively lower levels of Site B are found in neonatal, immature, and mature uteri (as compared to fetal uterus) which contain also decreasing amounts of estrogen receptors.

It is concluded that, besides binding to the estrogen receptor, the triphenylethylene antiestrogens bind to a specific site distinct from the estrogen receptor, that appears to be localized mainly in estrogen target cells.

INTRODUCTION

The triphenylethylene derivative tamoxifen (ICI 46474) has been shown to interact with estrogen target cells. It is well known that this compound is efficient in the remission of human breast cancer, especially estrogen receptor positive (44), and it is also capable of inhibiting cellular growth of cultured human cancer cells which have significant amounts of estrogen receptors (16, 24). Tamoxifen shows also agonistic and antagonistic estrogenic properties depending upon experimental conditions (16, 20, 21, 41). The estrogen agonistic properties of tamoxifen have been explained by its binding to the estrogen receptor and the consequent translocation of the complex into the nucleus. More controversial are the mechanisms supporting the estrogen antagonistic properties and the inhibitory effect on cellular growth of human mammary cancer cells in culture, even though the competition for the estrogen receptor system has also been suggested (16, 24). The responsiveness of the fetal uterus of guinea pig to estrogens has been reported previously (15, 29, 37). In this fetal organ, tamoxifen elicits a trophic effect similar to that caused by estradiol with a more limited stimulatory action on progesterone receptor synthesis as compared to estradiol (14). These responses appear to be related to the interaction with estrogen receptors (14, 15, 37) that are present at very high concentrations in this fetal organ (31). The use of tritiated tamoxifen allowed us to show the binding of tamoxifen to not only the fetal uterine estrogen receptor (Site A) but also a saturable binding site distinct from estrogen receptor, identified as Site B (14). The aim of the present paper is to investigate further the interaction of tamoxifen with the fetal uterus as well as to compare the binding characteristics of tamoxifen to the estrogen receptor and Site B, in order to emphasize differences and similarities between this antiestrogen binding site and the estrogen receptor system.

MATERIALS AND METHODS

Biological Material

Hartley albino guinea pigs (fetuses from 55 to 65 days of gestation and 7-day-old, 30-day-old, and adult animals) were used.

Chemicals

[6,7-^{3}H]Estradiol [1,3,5(10)-estratriene-3,17β-diol; 51 Ci/mmol] was obtained from New England Nuclear (Frankfurt, W. Germany) and used without further purification, since the purity was found to be greater than 95% in the appropriate paper chromatographic systems. [^{3}H]Tamoxifen [*trans*-1-(*p*-dimethylaminoethoxyphenyl)-1,2-*trans*-diphenylbut-1-ene; 15 Ci/mmol; 97% pure] was a gift from Dr. J. Burns (Imperial Chemical Industries, England); it was stored in the dark at −20°, and its purity was checked periodically by chromatography on thin-layer plates (Merck; 0.25-mm silica gel with fluorescent indicator) using benzene:triethylamine (9:1, v/v) as solvents. Tamoxifen (ICI 46474), tamoxifen Metabolites A (ICI 46929), B (monohydroxytamoxifen; ICI 79280), D (dihydroxytamoxifen; ICI 77307), and X (*N*-desmethyltamoxifen; ICI 55548) were gifts from Dr. L. Lacomme (ICI Pharma, Paris, France). Nafoxidine (1-[2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl] pyrrolidine hydrochloride) U 11, 100 A was a gift from Dr. J. P. Paturaud (Upjohn Laboratories, Paris, France). RU 16117[11α-methoxy-17-ethynyl-1,3,5(10)-estratriene-3,17β-diol]was

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a gift from Dr. J. P. Raynaud (Roussel-Uclaf, Romainville, France). Diethylstilbestrol and other steroids were purchased from Steraloids (Touzart et Matignon, Vitry-sur-Seine, France). Pronase (45,000 units/g) was obtained from Calbiochem-Behring Corp.

Preparation of Cytoplasmic Extracts

Uteri, hearts, and lungs, stripped of adhering fat and rinsed with ice-cold 0.9% NaCl solution, were homogenized in 10 mM Tris-HCl:1.5 mM EDTA:0.5 mM dithiothreitol (pH 7.4) with a Teflon:glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 900 × *g* for 10 min, and the supernatant was centrifuged again at 170,000 × *g* (average) for 30 min to obtain a clear cytosol supernatant. All procedures were carried out at 4°.

Single-Saturating-Dose Assay of Sites A and B and Estrogen Receptor

The specific binding of [^{3}H]tamoxifen was determined after incubation with [^{3}H]tamoxifen (15 nM) ± a 100-fold molar excess of unlabeled estradiol (for Site A assay) or [^{3}H]tamoxifen (15 nM) plus estradiol (2 μM) ± a 100-fold molar excess of unlabeled tamoxifen (for Site B assay) for 1 or 16 hr at 4°. The unbound [^{3}H]tamoxifen was removed by 0.1% (w/v) dextran-coated 1% (w/v) charcoal (final concentration) (DCC)[3] for 10 min at 4°. Under these conditions, most of the unbound ligand was adsorbed by 2 min with stable values of bound radioactivity (both specific and nonspecific) from 5 to at least 30 min.

The specific binding of [^{3}H]estradiol to the estrogen receptor in the cytosol was determined by 2 methods. (*a*) The cytosol was incubated with [^{3}H]estradiol (5 nM) ± a 100-fold molar excess of unlabeled estradiol for 1 or 16 hr at 4°. The unbound [^{3}H]estradiol was adsorbed by 0.05% (w/v) dextran-coated 0.5% (w/v) charcoal for 10 min at 4°. (*b*) The binding of [^{3}H]estradiol to the estrogen receptor was assayed by the [^{3}H]estradiol exchange technique on protamine sulfate precipitates described previously (6, 36) by incubating with [^{3}H]estradiol (10 nM) ± a 100-fold molar excess of unlabeled estradiol for 16 hr at 30°.

Measurement of Association and Dissociation Rate Constants

Association Rate. [^{3}H]Tamoxifen (10 nM) with or without a 100-fold molar excess of estradiol (for association rate analysis of Site A) or [^{3}H]tamoxifen (10 nM) with or without a 100-fold molar excess of tamoxifen (for association rate analysis of overall saturable binding sites) was added to cytosol at 4°. For the association rate analysis of Site B, cytosol was incubated previously at 4° for 1 hr with estradiol (2 μM) (to saturate estrogen receptors), and then [^{3}H]tamoxifen (10 nM) with or without a 100-fold molar excess of unlabeled tamoxifen was added to cytosol at 4°. Time points were taken at 2-min intervals by treating 0.2-ml samples with DCC at 4°. The association rate constant (k_{+1}) was determined according to the method of Bouton and Raynaud (2) by the slope of the linear part of a plot of $(2.3/L_0 - R_0) \log (LR_0/L_0R)$ *versus* time of incubation, where L_0 and L are the concentrations of free [^{3}H]tamoxifen, and R_0 and R are free receptor at zero time and the time point, respectively. In the case of the association of [^{3}H]tamoxifen to estrogen receptor, the contribution of the dissociation reaction has been found to be nonsignificant (less than 10% difference in the value of k_{+1}) as determined by calculating k_{+1} from the differential equation $d(LR)/dt = k_{+1}(L)(R) - k_{-1}(LR)$, according to the method of Sanborn *et al.* (34). The slope was obtained by regression analysis to assure the best fit.

Dissociation Rate. The dissociation rates were measured by the isotopic dilution technique. Uterine cytosol was incubated overnight at 4° with [^{3}H]tamoxifen (8 nM) with or without tamoxifen (2 μM) or [^{3}H]tamoxifen (8 nM) with or without estradiol (2 μM) or with [^{3}H]-tamoxifen (8 nM) plus estradiol (2 μM) with or without tamoxifen (2 μM). After the incubation, a 1000-fold molar excess of unlabeled tamoxifen or estradiol (in 2 μl ethanol; final ethanol concentrations, less than 0.1%) was added at either 4 or 26°, and at different times, 0.2-ml samples were treated with DCC at 4° to determine bound radioactivity. Each value was corrected by subtracting nonspecific binding. The dissociation rate constant (k_{-1}) is given by the slope of the line $k_{-1}\,t = \ln B/B_0$, where B_0 and B represent bound [^{3}H]tamoxifen at zero time and at time t, respectively. Regression analyses were performed to obtain the best fit.

Binding of [^{3}H]Tamoxifen Cytoplasmic Complexes to Isolated Nuclei

Fetal uteri were homogenized in TDS buffer with a Teflon:glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 900 × *g* for 10 min, and the supernatant was centrifuged again at 170,000 × *g* (average) for 30 min to obtain the cytosol fraction. A purified nuclear fraction was obtained according to the method of Chauveau *et al.* (7) as modified by Pasqualini *et al.* (30). The 900 × *g* pellet was homogenized in 10 mM Tris-HCl:3 mM $CaCl_2$:0.4 M sucrose, pH 7.4, and centrifuged at 900 × *g* for 10 min. The pellet was homogenized in 10 mM Tris-HCl:3 mM $CaCl_2$:2 M sucrose, pH 7.4, and centrifuged at 36,000 rpm for 60 min in a Beckman SW 40 rotor to obtain the purified nuclei, which were then resuspended in TDS buffer. This nuclear preparation appeared to be free of cytoplasmic contamination as assessed by light microscopy.

In order to investigate the binding of [^{3}H]tamoxifen cytoplasmic complexes to purified nuclei, the cytosol was incubated with [^{3}H]tamoxifen (15 nM) or [^{3}H]tamoxifen (15 nM) plus estradiol (2 μM) or [^{3}H]tamoxifen (15 nM) plus tamoxifen (2 μM) 1 hr at 4°. After 1 hr, 0.5 ml of the incubated cytosol was added to the nuclear fraction (0.3 ml) and incubated 60 min at 4°. This incubation was then heated at 30° for 15 and 60 min. At the end of each incubation, the tubes were kept in ice for 10 min and centrifuged at 900 × *g* for 10 min to separate the nuclear fraction, which was washed once with 10 mM Tris-HCl:3 mM $CaCl_2$:0.4 M sucrose (pH 7.4) buffer and again twice with TDS buffer. The radioactive material bound to the nuclei was then extracted with 10 mM Tris-HCl:0.6 M KCl:1.5 mM EDTA:0.5 mM dithiothreitol (pH 8.5) for 30 min at 4°. The binding of [^{3}H]tamoxifen to the cytosol was assayed by the DCC method described above.

Affinity of Binding of [^{3}H]Tamoxifen to Cytosol and Nuclear Macromolecules

The affinity of [^{3}H]tamoxifen binding in the cytosol and 0.6 M KCl nuclear extract was determined according to the method of Scatchard (35). Details are given in the legends of charts.

Protein and DNA Assay

Protein was measured by the method of Lowry *et al.* (25), and DNA was determined according to the method of Burton (4).

Statistical Analysis

All results are expressed as mean ± S.D. The Student *t* test was used to compare the means.

RESULTS

Validation of the Assay of [^{3}H]Tamoxifen-binding Sites. Chart 1 shows the saturation curves of [^{3}H]tamoxifen binding in the cytosol of fetal uterus in the absence or in the presence of a 100-fold molar excess of estradiol or tamoxifen or estradiol plus tamoxifen. As indicated, 2 saturable components are obtained (Chart 1*B*): the first one obtained after subtraction of the binding curve of [^{3}H]tamoxifen plus estradiol from that of [^{3}H]tamoxifen alone (Site A) and the second one found after subtraction of the nonsaturable binding of [^{3}H]tamoxifen (▼ or

[3] The abbreviations used are: DCC, dextran-coated charcoal; TDS buffer, 10 mM Tris-HCl:0.5 mM dithiothreitol:0.25 M sucrose (pH 7.4).

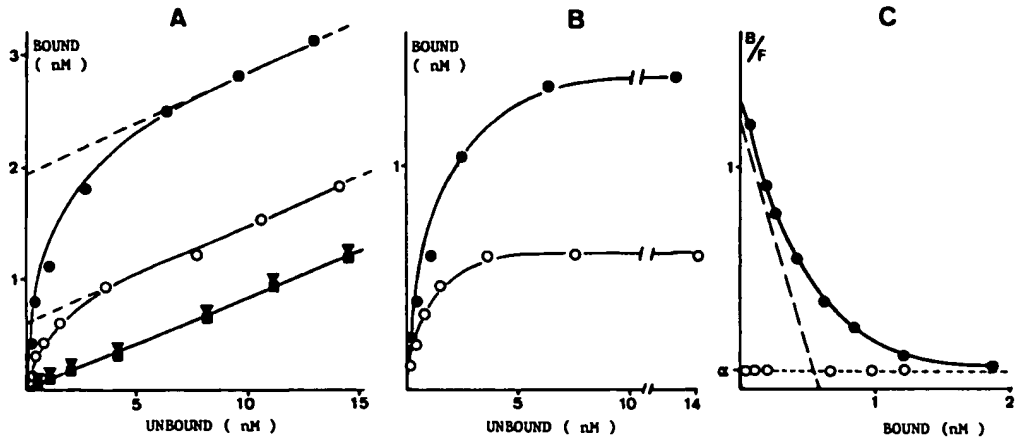

Chart 1. Saturation analysis of [^{3}H]tamoxifen binding in the fetal uterine cytosol. *A*, uterine cytosol incubated overnight at 4° with [^{3}H]tamoxifen (from 20 pM to 16 nM) (●) or with the same range of concentrations of [^{3}H]tamoxifen plus estradiol (2 μM) (○) or plus tamoxifen (2 μM) (▼) or plus tamoxifen (2 μM) and estradiol (2 μM) (■). After the incubation, unbound radioactivity was adsorbed by DCC, and the concentrations of bound [^{3}H]tamoxifen were plotted against the concentrations of unbound radioactive ligand. *B*, saturable components of binding parameters represented in *A* obtained after subtraction of binding curve of [^{3}H]tamoxifen plus estradiol from that of [^{3}H]tamoxifen alone (Site A, ●) and after subtraction of binding curve of [^{3}H]tamoxifen plus estradiol and tamoxifen from that of [^{3}H]tamoxifen plus estradiol (Site B, ○). *C*, Scatchard plot of the data represented in *A*. ●, the binding of [^{3}H]tamoxifen in the presence of estradiol (2 μM); ○, the binding of [^{3}H]tamoxifen in the presence of estradiol (2 μM) and tamoxifen (2 μM). The 2 linear components of the curve (— — —, - - - -) were obtained by the method of Rosenthal (33). α is the intercept on the *y* axis, corresponding to the slope of the linear portion of the binding curves in *A*. *B*, bound; *F*, free.

■) from the binding curve of [^{3}H]tamoxifen plus estradiol (Site B). Furthermore, the curve of the nonsaturable binding of [^{3}H]tamoxifen (evaluated experimentally by the incubation containing a 100-fold molar excess of unlabeled tamoxifen or tamoxifen plus estradiol, with respect to the radioactive ligand) is linear over the range of concentrations used and shows the same slope (α, 0.08 in the experiment represented) as does the nonsaturable binding of [^{3}H]tamoxifen extrapolated as the straight line from the nonsaturable component of the binding curves of the incubations containing [^{3}H]tamoxifen alone or [^{3}H]tamoxifen plus estradiol (Chart 1*A*). This shows that the nonsaturable binding of [^{3}H]tamoxifen is unchanged in the different conditions of assay. The identity of the nonsaturable binding of [^{3}H]tamoxifen in the different conditions of assay was further demonstrated by the Scatchard analysis of binding parameters shown in Chart 1C. In this chart, the binding curve of [^{3}H]tamoxifen plus unlabeled estradiol (2 μM) has been resolved by the method of Rosenthal (33) into 2 lines. The first one is saturable (*broken line*), and the second one is a nonsaturable line (*horizontal broken line*), which expresses a linear relationship between bound and unbound [^{3}H]tamoxifen concentration, with intercept α on the *y* axis. The nonsaturable binding of [^{3}H]tamoxifen experimentally evaluated by incubations with [^{3}H]tamoxifen plus a 100-fold molar excess of unlabeled tamoxifen and estradiol is represented by the *open symbols* that describe a horizontal line; this line also intercepts the *y* axis on α.

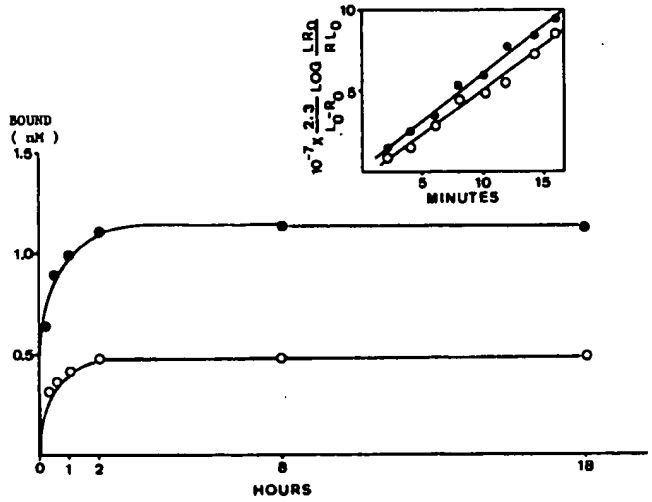

Chart 2. Curves of the formation of [^{3}H]tamoxifen:Site A and [^{3}H]tamoxifen:Site B complexes as a function of incubation time. Fetal uterine cytosol was incubated at 4° with [^{3}H]tamoxifen (10 nM) with or without estradiol (2 μ for Site A assay (●). For Site B assay (○), the cytosol was incubated previously 1 hr at 4° with estradiol (2 μM) and then with [^{3}H]tamoxifen (10 nM) with or without a 100-fold molar excess of unlabeled tamoxifen, as described previously. Incubations were stopped at the indicated times by adsorption of unbound ligand with DCC. The *inset* represents the association rate constants of formation of [^{3}H]tamoxifen:Site A (●) and [^{3}H]tamoxifen:Site B (○) complexes, calculated by the slopes of the lines represented as described in "Materials and Methods."

Association and Dissociation Kinetics of [^{3}H]Tamoxifen Binding in Fetal Uterine Cytosol. Chart 2 shows the binding of [^{3}H]tamoxifen to Sites A and B at 4° as a function of incubation time. As indicated, maximal binding of [^{3}H]tamoxifen was attained between 1 and 2 hr for both Sites A and B, with stable values up to 18 hr.

The association rate constant (k_{+1}) of [^{3}H]tamoxifen to Sites A and B is also represented (Chart 2, *inset*). As indicated, similar values were found for both Sites A and B (k_{+1} 1.03 ± 0.5 and k_{+2}, 0.95 ± 0.16 × 10^5 M^{-1} sec^{-1}, respectively). The overall association of [^{3}H]tamoxifen to saturable cytoplasmic binding sites (assumed as a second-order reaction) was similar (k_{+1} 0.94 ± 0.19 × 10^5 M^{-1} sec^{-1}).

The dissociation of [^{3}H]tamoxifen from the cytoplasmic binding sites was determined by adding unlabeled tamoxifen, which allows the measurement of the dissociation of [^{3}H]tamoxifen from both Sites A and B. The dissociation at 4° occurred in 2 exponential phases (Chart 3*A*, ——); the dissociation rate constant of the first more rapid component (k_{-1}), after subtraction of the second slower dissociation component (Chart 3*A*, *inset*), was 11.43 ± 0.6 × 10^{-4} sec^{-1}. The dissociation rate

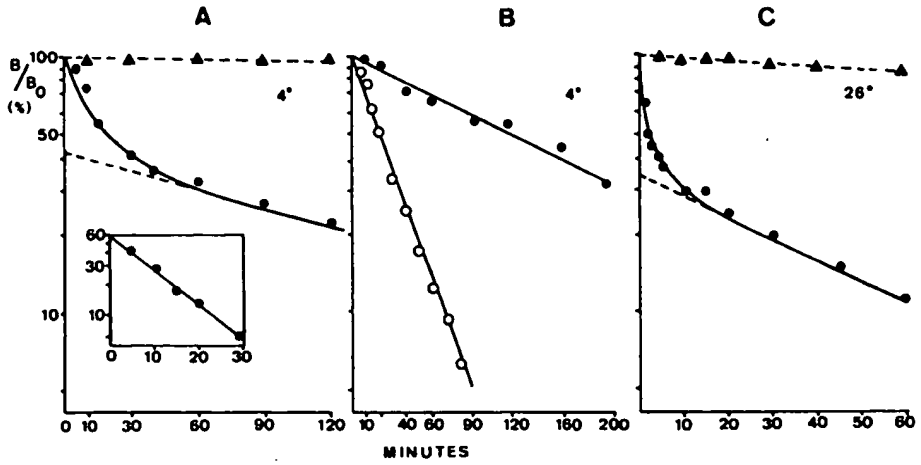

Chart 3. Dissociation of [^{3}H]tamoxifen from fetal uterine cytoplasmic binding sites. Dissociation rates were measured by the isotopic dilution technique as described in "Materials and Methods." The same procedure, without adding unlabeled ligand, was used to measure binding site stability (▲). *A*, uterine cytosol incubated overnight at 4° with [^{3}H]tamoxifen (8 nM) with or without a 100-fold molar excess of unlabeled tamoxifen. The dissociation of [^{3}H]tamoxifen at 4° was determined after addition of unlabeled tamoxifen. The rate constant of the first component was estimated after removing the contribution of the second component extrapolated to zero time (*inset*) according to the method of Weichman and Notides (42). *B*, dissociation rate of [^{3}H]tamoxifen from Sites A (○) and B (●) at 4°. For the determination of the dissociation of [^{3}H]tamoxifen from Site A, uterine cytosol was incubated overnight at 4° with [^{3}H]tamoxifen (8 nM) with or without a 100-fold molar excess of unlabeled estradiol, and the dissociation process was started by addition of unlabeled estradiol (○). For the analysis of the dissociation rate of [^{3}H]tamoxifen from Site B (●), uterine cytosol, incubated overnight at 4° with [^{3}H]tamoxifen (8 nM) with or without unlabeled tamoxifen (2 μM), was reincubated with a 500-fold molar excess of unlabeled estradiol, and after 3 hr (considered zero time), when practically all the [^{3}H]tamoxifen was dissociated from estrogen receptor, unlabeled tamoxifen was added to dissociate further the radioactive ligand. *C*, dissociation rate of [^{3}H]tamoxifen at 26°. The experimental procedure has been described in *A*.

constant of the slow component (k_{-2}) was 0.95 ± 0.26 × 10^{-4} sec^{-1}. In order to investigate whether one of the 2 exponential phases represented the dissociation of [^{3}H]tamoxifen from the estrogen receptor (Site A) and the other from Site B, unlabeled estradiol was used to bring about the dissociation of [^{3}H]tamoxifen from the Site A alone (Chart 3*B*, ○). The resulting dissociation rate constant (k_{-1}) was 8.3 ± 2 × 10^{-4} sec^{-1}. The dissociation of [^{3}H]tamoxifen from Site B was investigated after first displacing [^{3}H]tamoxifen from Site A by unlabeled estradiol at 4°. The subsequent addition of unlabeled tamoxifen provokes a dissociation of [^{3}H]tamoxifen with a rate constant (k_{-2}) of 0.81 ± 0.14 × 10^{-4} sec^{-1} (Chart 3*B*, ●). Similar results were obtained when the binding of [^{3}H]tamoxifen to Site A was prevented by previous incubation with unlabeled estradiol (2 μM) (as described in "Materials and Methods" for the assay of Site B), and unlabeled tamoxifen was used to dissociate the [^{3}H]tamoxifen:Site B complex. Thus, it is concluded that the more rapidly dissociating component represents the estrogen receptor and that the more slowly dissociating one, the Site B. Since the association rate constant (k_{+1}) of [^{3}H]tamoxifen to both Sites A and B is similar and the same as the overall association rate constant of [^{3}H]tamoxifen, the association constant (K_a) of the binding of [^{3}H]tamoxifen was calculated from the k_{+1}:k_{-1} and k_{+1}:k_{-2} ratios, respectively. The results indicate that [^{3}H]tamoxifen binding has 2 affinity states which agrees with the data obtained previously by Scatchard plots under equilibrium conditions (14).

Increasing the incubation temperature (26°) accelerates the dissociation rate of [^{3}H]tamoxifen from cytoplasmic binding sites (Chart 3*C*). The first phase of dissociation (after subtraction of the second slowly dissociating component) was very fast (k_{-1} 123 ± 26 × 10^{-4} sec^{-1}) and corresponds to the dissociation of [^{3}H]tamoxifen from Site A, since similar results were obtained using unlabeled estradiol to bring about the dissociation of [^{3}H]tamoxifen. Similar values of the dissociation rate constant at 25° have already been reported for the estrone:estrogen receptor complex (43). The dissociation rate constant of the slower component (k_{-2}) was 3.0 ± 0.4 × 10^{-4} sec^{-1}.

A similar great temperature dependence has been described previously for the dissociation of estrogenic compounds from the fetal uterine estrogen receptor. The dissociation rate constant of the estradiol:estrogen receptor complex is 2.2 × 10^{-6} sec^{-1} and 1.93 × 10^{-4} sec^{-1} at 4 and 25°, respectively, and that of the estriol:estrogen receptor complex is 3.7 × 10^{-6} sec^{-1} at 4° and 6.0 × 10^{-4} sec^{-1} at 25° (15), according to results found by other workers (5, 22, 43). In contrast, it is interesting to note that the dissociation of the [^{3}H]-tamoxifen:Site B complex shows a lesser temperature dependence.

Competition by Several Steroids and Triphenylethylene Derivatives for Site B and Estrogen Receptor in the Fetal Uterus. The competitive effect of different steroids and triphenylethylene derivatives on the [^{3}H]tamoxifen binding Site B was studied in the presence of unlabeled estradiol (2 μM) to saturate all estrogen receptors (Chart 4*A*). As indicated, progesterone, cortisol, and testosterone do not inhibit the specific binding of [^{3}H]tamoxifen. No competition was observed using the nonsteroidal estrogen, diethylstilbestrol. RU 16117, a synthetic estrogen derivative with antiestrogenic properties (3), does not compete either for [^{3}H]tamoxifen binding. In contrast, nafoxidine and other triphenylethylene derivatives (Chart 4, *C* to *E*) compete for the [^{3}H]tamoxifen binding, with a relative binding affinity similar (nafoxidine) or lower (Tamoxifen Metabolites A, B, D, and X) than tamoxifen (Table 1). This inhibition of the binding of [^{3}H]tamoxifen to Site B has been shown to be competitive, as indicated by the common *y* intercept in the competition plot shown in Chart 4*B*, suggesting a common site for all these triphenylethylene compounds. Table 1 shows also the competition of the above described triphenylethylene derivatives for the estrogen receptor. As indicated, the introduction of hydroxyl groups in the phenyl ring greatly increases the

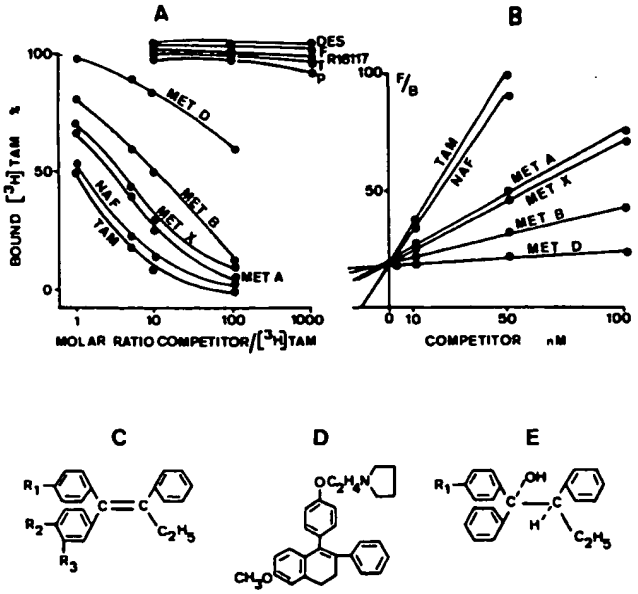

Chart 4. Competitive effect of some steroids and triphenylethylene derivatives on [^{3}H]tamoxifen binding to cytoplasmic Site B in the fetal uterus. *A*, aliquots of uterine cytosol incubated with [^{3}H]tamoxifen (10 nM) plus estradiol (2 μM) with or without different concentrations of diethylstilbestrol (*DES*), cortisol (*F*), R 16117, testosterone (*T*), progesterone (*P*), tamoxifen (*TAM*), tamoxifen Metabolite A (*MET A*), monohydroxytamoxifen (*MET B*), dihydroxytamoxifen (*MET D*), *N*-desmethyltamoxifen (*MET X*), and nafoxidine (*NAF*) for 16 hr at 4°. The saturable binding of [^{3}H]tamoxifen (determined by the DCC method) is represented. *B*, uterine cytosol incubated with [^{3}H]tamoxifen plus unlabeled estradiol as described in *A*, in the absence or in the presence of different concentrations of the compounds indicated. The data were analyzed and represented according to the method of Munck (27), plotting the concentration of the competitor *versus* the ratio between unbound and bound [^{3}H]tamoxifen (*F/B*). The results represent the mean from 2 to 3 experiments. *C*, tamoxifen, R_1 = $(CH_3)_2NCH_2CH_2O$; R_2 and R_3 = H. Monohydroxytamoxifen, R_1 = $(CH_3)_2NCH_2CH_2O$; R_2 = OH; R_3 = H. Dihydroxytamoxifen, R_1 = $(CH_3)_2NCH_2CH_2O$; R_2 = OH; R_3 = OH. *N*-desmethyltamoxifen, R_1 = $CH_3NHCH_2CH_2O$; R_2 and R_3 = H. *D*, nafoxidine (U 11100 A). *E*, ICI 46929 (Metabolite A), R_1 = $(CH_3)_2NCH_2CH_2O$.

Table 1

Relative binding affinity of several triphenylethylene derivatives for Site B and estrogen receptor in the fetal uterus

The relative binding affinity of the different competitors for Site B was investigated in the presence of [^{3}H]tamoxifen (10 nM) plus estradiol (2 μM) using a competitor concentration range of 1 nM to 10 μM. Competition studies for estrogen receptor were carried out using [^{3}H]estradiol (2 nM) and a competitor concentration range of 0.2 nM to 2 μM. The relative affinity values depicted equal the competitor association constants (calculated by the competition plot described in Chart 1*B*) divided by the association constant of [^{3}H]tamoxifen for Site B or [^{3}H]estradiol for estrogen receptor, respectively. The values represent the mean from 2 experiments.

	Relative binding affinity (%)	
Competitor	Site B	Estrogen receptor
Estradiol	0	100
Tamoxifen	100	9
Nafoxidine	90	15
Metabolite A (ICI 46929)	35	0.3
Monohydroxytamoxifen	10	90
Dihydroxytamoxifen	0.5	50
N-Desmethyltamoxifen	32	9

affinity for the estrogen receptor. In contrast, the hydroxylation and saturation of the ethylene chain interconnecting the phenyl rings dramatically decrease the affinity for the estrogen receptor, as already described for MER-25 and WSM-4613 (41).

Effect of Proteolytic Treatment on [^{3}H]Estradiol and [^{3}H]-Tamoxifen Binding in the Fetal Uterine Cytosol. Uterine cytosol containing a saturating concentration of [^{3}H]estradiol or [^{3}H]tamoxifen with or without a 100-fold molar excess of unlabeled ligand was treated with Pronase (45 units/ml) for 2 hr at 4°, according to the method of Capony and Rochefort (5), or for 1 hr at 30°, and the saturable binding was evaluated by the DCC assay. This treatment totally destroyed either the specific binding of [^{3}H]estradiol or the binding of [^{3}H]tamoxifen to the estrogen receptor protein. Similarly, Site B (evaluated by the binding of [^{3}H]tamoxifen in the presence of unlabeled estradiol) was totally destroyed by this treatment, suggesting that this binding is also supported by proteins.

Effect of Ammonium Sulfate Precipitation on Cytoplasmic Tamoxifen Sites A and B. Sequential precipitation of [^{3}H]-tamoxifen-labeled cytosol was carried out using different percentages of saturation of ammonium sulfate, as indicated in Chart 5. The results showed that 90% of Sites A and B was precipitated by concentrations of ammonium sulfate up to 48%, with most of the sites being precipitated in the 36% fraction. Furthermore, the precipitation profile of both Sites A and B was similar.

Thermal Stability of [^{3}H]Estradiol and [^{3}H]Tamoxifen-binding Sites in Fetal Uterine Cytosol. It is well known that the heating of the unfilled estrogen receptor inhibits its subsequent binding of estradiol. This phenomenon has also been found for fetal uterine estrogen receptor, since the heating at 37° for 1 hr of uterine cytosol in the absence of the hormone almost totally destroyed the specific binding of [^{3}H]estradiol or the binding of [^{3}H]tamoxifen displaced by unlabeled estradiol (*i.e.*, its binding to the estrogen receptor). On the contrary, the binding of tamoxifen to Site B was not affected by this thermal treatment, suggesting that Site B is thermoresistant (data not shown).

***In Vitro* Binding of [^{3}H]Tamoxifen Cytoplasmic Complexes to Isolated Nuclei of the Fetal Uterus.** In most steroid receptor systems, the cytoplasmic receptor, after having bound the hormone, translocates into the nucleus by a process that is mediated by temperature, *in vitro*. The ability of [^{3}H]tamoxifen cytoplasmic complexes to bind to uterine nuclei was tested

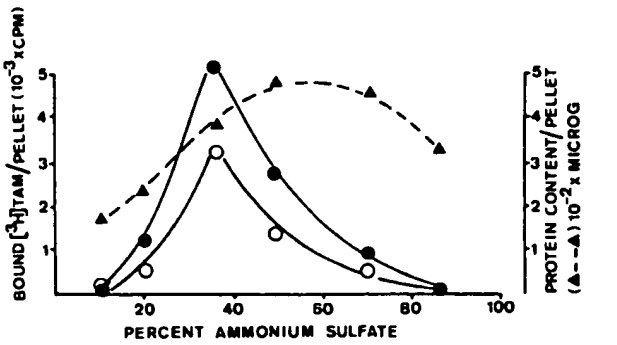

Chart 5. Precipitation of tamoxifen Sites A (●) and B (○) by ammonium sulfate. Fetal uterine cytosol was incubated with [^{3}H]tamoxifen ([^{3}*H*]*TAM*) (10 nM) ± a 100-fold molar excess of unlabeled estradiol (for Site A assay) or [^{3}H]tamoxifen (10 nM) plus unlabeled estradiol (2 μM) ± a 100-fold molar excess of unlabeled tamoxifen (for Site B assay), at 4° overnight, after which the unbound ligand was adsorbed by DCC, and sequential ammonium sulfate precipitations were performed at 4° with the indicated percentages of saturation of ammonium sulfate. The precipitate was collected by centrifugation at 15,000 × *g* for 10 min and redissolved in TED buffer for counting of radioactivity, while the supernatant was submitted to the next precipitation. Protein concentration in each ammonium sulfate-precipitated fraction was quantitated according to the method of Lowry *et al.* (25). The results shown are the mean of 2 distinct duplicate analyses.

using a cell-free system, as shown in Chart 6. The estrogen receptor:tamoxifen complex does not bind to uterine nuclei at 2°, but this binding occurs at 30°, as shown by the decrease of bound radioactivity in the cytosol with the concomitant increase in the nucleus. On the contrary, the tamoxifen:Site B complex does not bind to uterine nuclei because no decrease of bound radioactivity was observed in the cytosol fraction, and no increase was observed in the nuclei. Thin-layer chromatography of the radioactive material extracted by 0.6 M KCl from the nuclear fraction of the uterine tissue showed that it was represented mostly by unmetabolized [^{3}H]tamoxifen (90 to 95%).

However, a saturable binding of [^{3}H]tamoxifen was found in the nuclei, both at 2 and 30°; this binding was probably not mediated by cytoplasmic proteins because it was observed even in the absence of cytosol, and it did not increase with increasing amounts of cytosol (not shown).

In order to investigate this direct binding of [^{3}H]tamoxifen to uterine nuclei, Scatchard analyses were carried out in the 0.6 M KCl nuclear extract in the presence of unlabeled estradiol (2 μM) to eliminate any possible binding to estrogen receptor.

As indicated in Chart 7, 2 classes of binding sites were found. (*a*) One had a binding affinity (K_d 0.47 ± 0.1 nM) similar to that of cytoplasmic tamoxifen Site B and a concentration of 98 ± 20 fmol/mg protein (0.34 ± 007 pmol/mg DNA). (*b*) The other had a much lower affinity (K_d 12.5 ± 2 nM) and a concentration of 326 ± 60 fmol/mg protein (1.1 ± 0.2 pmol/mg DNA). DCC has been used to evaluate the binding of [^{3}H]tamoxifen in the 0.6 M KCl nuclear extract. Since DCC has been described to strip estradiol from its receptor in the presence of 0.6 M KCl (32), the stability of the binding of [^{3}H]-tamoxifen to cytoplasmic Site B as evaluated by the DCC assay was investigated in the presence of increasing amounts of KCl from 0 to 0.6 M. The results showed that DCC did not strip [^{3}H]tamoxifen from Site B in the presence of KCl.

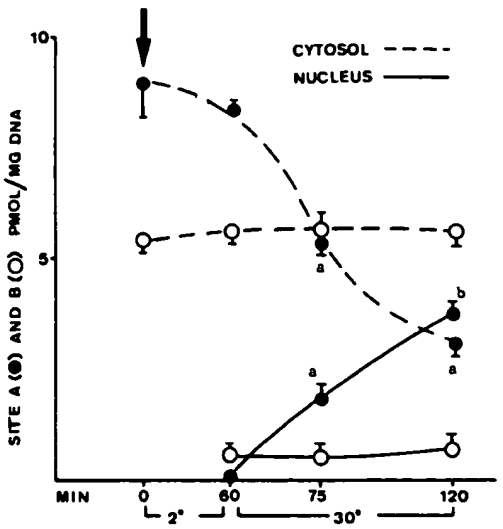

Chart 6. Binding of [^{3}H]tamoxifen cytoplasmic complexes to isolated nuclei in the fetal uterus. Cytoplasmic and nuclear fractions were prepared as described in "Materials and Methods." Aliquots of the cytosol fraction were incubated 60 min at 2° with [^{3}H]tamoxifen (15 nM) with or without estradiol (2 μM) to determine its binding to the estrogen receptor (Site A) (●) and with [^{3}H]tamoxifen (15 nM) plus estradiol (2 μM) with or without unlabeled tamoxifen (2 μM) to determine its binding to Site B (○). The cytoplasmic complexes so formed were incubated for 60 min at 2° with the nuclear fraction and then for 15 or 60 min at 30°. The binding of the tamoxifen:estrogen receptor (Site A) complex (●) and tamoxifen:Site B complex (○) in the nucleus was evaluated as described in "Materials and Methods." The binding of [^{3}H]tamoxifen to the cytosol was assayed by the DCC method. The *arrow* indicates the introduction of the nuclear fraction. The results represent the mean ± S.D. from 3 distinct triplicate experiments. *a*, $p \leq 0.01$; *b*, $p < 0.001$ (*versus* zero time for cytosol and 60 min for nuclei).

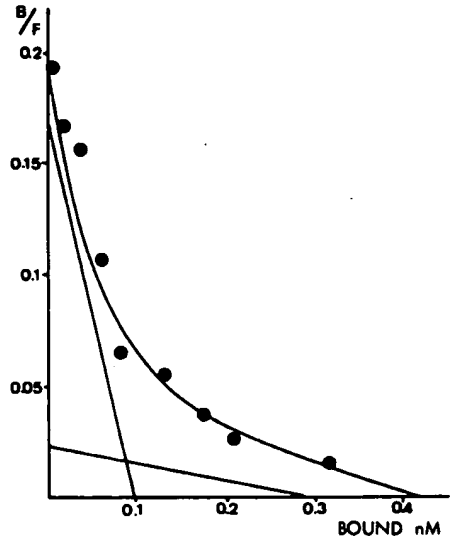

Chart 7. Scatchard analysis of [^{3}H]tamoxifen binding in the 0.6 M KCl nuclear extract from the fetal uterus. Aliquots of the fetal uterine 0.6 M KCl nuclear extract (containing 1 to 1.3 mg protein per ml) were incubated overnight at 4° with [^{3}H]tamoxifen (50 pM to 15 nM) plus estradiol (2 μM) ± a 100-fold molar excess of unlabeled tamoxifen. Binding was determined using the DCC method. The saturable binding is depicted. The method of Rosenthal (33) for the resolution of the 2 binding sites has been used. *B/F*, bound:free.

It is concluded that tamoxifen can translocate the estrogen receptor to the nucleus by a temperature-dependent process, while it cannot translocate its specific binding site.

Distribution of Tamoxifen Sites A and B in Fetal Heart and Lung and in Fetal, Neonatal, Immature, and Mature Uterus. In order to investigate a possible relationship between Site B and estrogen receptor, its presence was examined in fetal organs considered nontarget for estrogen action and which show no estrogen receptors (heart) or very low levels (lung, 20 ± 1.0 fmol/mg protein) (31).

Chart 8*A* shows a Scatchard analysis of [^{3}H]tamoxifen-saturable binding in the presence of unlabeled estradiol (2 μM) in cytosol of fetal heart. As indicated, [^{3}H]tamoxifen binds to a single class of sites with a K_d of 0.6 ± 0.15 nM similar to Site B of uterine cytosol but with a concentration significantly ($p < 0.001$) lower (103 ± 50 fmol/mg protein). Similar analyses carried out in the cytosol of fetal lung (Chart 8*B*) in the presence of estradiol (2 μM) show that [^{3}H]tamoxifen binds to 2 classes of sites. The first one has a K_d of 0.49 ± 0.13 nM, similar to uterine Site B, but with a concentration significantly lower ($p < 0.001$) (127 ± 60 fmol/mg protein). The second one has a K_d of 6.88 ± 0.33 nM and a concentration of sites of 317 ± 42 fmol/mg protein. This second class of sites could be due to plasmatic contamination, because a binding for tamoxifen with a similar K_d was found in the fetal plasma of guinea pig (14).

Since uterine estrogen receptors have been shown to reach highest levels during fetal life with decreasing values after birth up to the adult period (31, 36), the evolution of uterine Site B in different periods of life was also investigated and correlated with estrogen receptors.

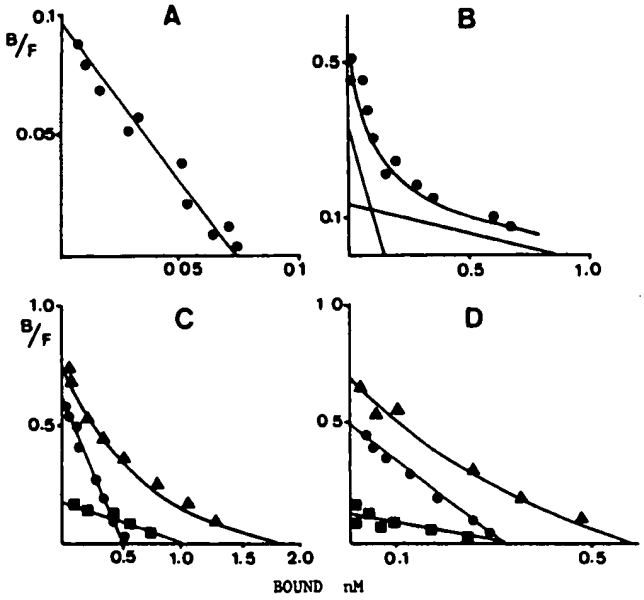

Chart 8. Scatchard analysis of [^{3}H]tamoxifen binding in the cytosol of fetal heart (*A*) and lung (*B*) and neonatal (*C*) and adult (*D*) uterus. Aliquots of cytosol fraction (containing 1 to 2 mg protein per ml) were incubated overnight at 4° with [^{3}H]tamoxifen (50 pM to 15 nM) with or without unlabeled tamoxifen (2 μM) (▲), [^{3}H]tamoxifen (50 pM to 15 nM) plus estradiol (2 μM) with or without unlabeled tamoxifen (2 μM) (●), and [^{3}H]tamoxifen (50 pM to 15 nM) with or without estradiol (2 μM) (■). Binding was determined using the DCC method. The saturable binding is depicted. The 2 binding sites in *B* were resolved by the method of Rosenthal (33). *B/F*, bound:free.

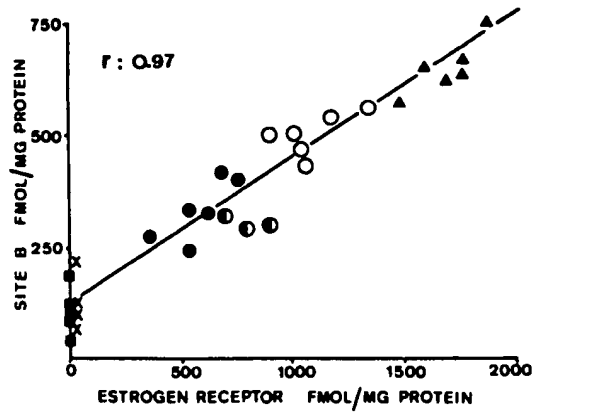

Chart 9. Distribution of tamoxifen Site B and estrogen receptor in different organs of guinea pig. Fetal (▲), neonatal (○), immature (◐), and adult (●) uteri and fetal hearts (■) and lungs (×) were processed for preparing the cytoplasmic fraction as described in "Materials and Methods." The levels of Site B were determined by Scatchard analysis or the single saturating dose assay described in "Materials and Methods," by incubating the cytosol at 4° overnight with [^{3}H]tamoxifen (15 nM) plus estradiol (2 μM) ± a 100-fold molar excess of unlabeled tamoxifen. The estrogen receptors were determined by incubating the protamine sulfate precipitates of the cytoplasmic fraction with a saturating amount of [^{3}H]estradiol (10 nM) ± a 100-fold molar excess of unlabeled estradiol under exchange conditions (30° for 16 hr) according to the method described previously (36). The regression line was calculated by the method of least-squares analysis ($y = 0.33x + 122$).

As reported previously, the Site B concentration in the fetal uterus was 655 ± 55 fmol/mg protein (K_d 0.39 ± 0.01 nM), while Site A showed a K_d of 1.8 ± 0.4 nM and a concentration of 1800 ± 100 fmol/mg protein (14). Chart 8*C* shows a Scatchard analysis of the [^{3}H]tamoxifen-saturable binding to the uterine cytosol of newborn guinea pigs. As indicated, in the neonatal uterus, [^{3}H]tamoxifen binds also to 2 classes of sites: Site A with a K_d of 5.5 ± 0.1 nM and a concentration of 1200 ± 61 fmol/mg protein, which is displaced by estradiol and which corresponds to estrogen receptor; and Site B with a K_d of 0.72 ± 0.05 nM and a concentration of 502 ± 40 fmol/mg protein that does not bind estradiol. Similar results arose from Scatchard analyses of [^{3}H]tamoxifen binding carried out in the cytosol of mature uterus that show a Site A concentration of 338 ± 81 fmol/mg protein and a K_d of 2.3 ± 0.1 nM and a Site B concentration of 327 ± 60 fmol/mg protein and a K_d of 0.71 ± 0.1 nM (Chart 8*D*).

Chart 9 shows the concentration of Site B in fetal, neonatal, immature, and adult uterus as well as fetal heart and lung as a function of estrogen receptor levels (evaluated by [^{3}H]estradiol binding). As indicated, a positive correlation ($r = 0.97$) was found between the levels of estrogen receptors and Site B. Similar results were found when data were expressed as amounts of binding sites per mg of DNA or per g of tissue, suggesting that this parallel change of estrogen receptors and Site B concentrations was not due to a change of cytoplasmic protein composition.

DISCUSSION

As reported previously (14), besides binding to the uterine estrogen receptor of the fetal guinea pig (Site A, K_d 1.8 nM), [^{3}H]tamoxifen binds also, with a higher affinity (K_d 0.39 nM), to a distinct binding site (Site B) that can be differentiated from the estrogen receptor by several properties (thermal sensitivity, dissociation rate constant, binding specificity, ability to bind to isolated nuclei).

Site B appears to be thermoresistant, while estrogen receptor is not. A similar thermal stability has been reported recently for a specific tamoxifen binding site insensitive to estradiol found in the uterine cytosol of mature rat (11).

The dissociation kinetics of [^{3}H]tamoxifen from uterine cytoplasmic binding sites also permits a differentiation between tamoxifen Sites A and B, the latter having a half-life longer than that of the [^{3}H]tamoxifen:Site A complex. It is interesting to note that the [^{3}H]tamoxifen:estrogen receptor complex dissociates more rapidly than does the [^{3}H]estradiol:estrogen receptor complex, similar to data found in 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors (28) and calf uterus (5). Similar results were also obtained for the uterine estrogen receptor of immature rat using another triphenylethylene derivative, CI 628 (22). The competition studies show that tamoxifen Site B appears to be insensitive not only to natural estrogens (14) but also to some synthetic estrogens with either estrogenic (diethylstilbestrol) or antiestrogenic (RU 16117) (3) activities. Androgens, progestins, and glucocorticoids also do not inhibit the binding of [^{3}H]tamoxifen to Site B. Only the compounds of the triphenylethylene type (nafoxidine, monohydroxytamoxifen, dihydroxytamoxifen, ICI 46929, *N*-des methyltamoxifen) show a competitive effect for Site B. Although these triphenylethylene derivatives also bind to the estrogen receptor, the structure competitive activity relationships of the tamoxifen metabolites show that the hydroxylation of the phenyl ring increases the affinity for estrogen receptor, while it decreases that for Site B. Thus, the stereospecificity of Site B appears to be very different from that of estrogen receptor.

A specific binding of tamoxifen insensitive to estradiol has

been reported recently by other authors in different tissues (11, 38, 40). Like the data obtained in the fetal uterus of guinea pig, this tamoxifen binding site also appears to be specific for the triphenylethylene derivatives. The binding affinity, evaluated indirectly by competition studies, of several triphenylethylene derivatives, such as nafoxidine (11, 40) and *N*-des methyltamoxifen (40), for the antiestrogen binding site in different experimental models appears to be similar to that for Site B. Nevertheless, some differences are reported for other triphenylethylene derivatives. For instance, CI 628 appears to have a lesser affinity than does tamoxifen for the antiestrogen binding site found in mature rat uterine cytosol (11), while it has the same affinity as does tamoxifen in MCF-7 cells (40). Also, monohydroxytamoxifen shows a lesser affinity, as compared to tamoxifen, for Site B (present study) than for the antiestrogen binding site in MCF-7 cells (40).

The affinity of tamoxifen for this triphenylethylene binding site is higher in the guinea pig as compared to the other experimental models, while that for the estrogen receptor is similar (11, 38, 40). All these results suggest the existence of a common binding site specific for the triphenylethylene antiestrogens, although probably, with structural species and tissue differences, which could explain the slight differences in specificity for the different triphenylethylene derivatives and in affinity for tamoxifen.

Another property that differentiates Sites A and B is the ability of the tamoxifen:estrogen receptor complex to translocate to the nucleus in a cell-free system, while the tamoxifen:Site B complex does not. This step, which generally occurs in the early stages of the interaction of any steroid hormone with its own receptor, is absent for Site B. A nuclear translocation of estrogen receptor by antiestrogens in the fetal uterus has also been shown after *in vivo* injection of tamoxifen and nafoxidine, which may account for the estrogenic responses elicited by these compounds (14). Previous studies have shown that, after injection of radioactive CI 628 to immature rat, this compound was not retained in uterine nuclei where a more polar metabolite could be isolated (22). The same authors also failed to demonstrate a nuclear translocation of estrogen receptor using [^{3}H]CI 628 *in vitro* up to 1 μM concentrations in calf uterus (9). Since tamoxifen, like CI 628, has been shown to be converted to several metabolites *in vivo* (12, 13), some of which have a higher affinity for estrogen receptor (Ref. 18; present study), it has been suggested that the metabolites formed *in vivo* from these triphenylethylene compounds are the biologically active forms of the antiestrogens. However, the present data show that tamoxifen itself, at only slightly more than saturating concentrations (15 nM), is capable of translocating the estrogen receptor into the nucleus *in vitro*. These results are in agreement with previous results reported in an estrogen- and antiestrogen-responsive human breast cancer cell line (MCF-7) in culture, where unmetabolized tamoxifen was accumulated in nuclei (16).

The present study also shows that Site B could be tissue specific because significantly higher concentrations of Site B were found in estrogen target tissues as compared to estrogen nontarget organs (heart and lung), in agreement with the tissue localization of the antiestrogen binding site reported by Sutherland *et al.* (40). However, the concentration of Site B appears to be significantly higher (2 to 7 times) in the fetal uterus as compared to the other estrogen target organs after birth (11, 38, 40). It is interesting to note that the fetal uterine estrogen receptor levels are also much higher than those found in different estrogen target tissues after birth (40). This phenomenon could suggest a correlation between the levels of estrogen receptor and Site B, as indeed supported by the concomitant decrease of estrogen receptors and Site B in the uterus from fetal to adult life (Chart 9). This relationship of Site B to estrogen receptor is also suggested by the recent report of a high-affinity saturable binding site for tamoxifen distinct from the estrogen receptor that is detectable only in estrogen receptor-positive but not in estrogen receptor-negative human mammary carcinoma cytosol (39). The nature of this relationship is unclear. However, it could be connected with the mechanisms that mediate sex hormone activity, as suggested by the cyclic variation of uterine antiestrogen binding site concentrations during the estrus cycle in the rat (11). Although Site B appears to be localized in estrogen target cells, it is interesting to note that low levels of this site are physiologically present also in tissues that lack or almost completely lack estrogen receptor (Ref. 40; present study). This suggests the possibility of a widespread role for antiestrogen binding sites, which is distinct from estrogenic action but to some extent modulated by the quantities of estrogen receptor or by the factors that control estrogen receptor levels.

Tamoxifen and other related compounds of the triphenylethylene class have been shown to elicit complex responses on estrogen target cells, such as estrogen-agonistic, partial agonistic, and -antagonistic actions as well as an inhibitory effect on cellular growth of human mammary cancer in culture. The competition of these compounds for the estrogen receptor has been suggested as an explanation for their estrogen-antagonistic properties, by interfering with the interaction of estrogen receptors with the nuclear chromatin (1, 17, 26) and/or the regeneration of the cytoplasmic receptor (8), even though the latter hypothesis is untenable for tamoxifen that appears to be capable of causing a full replenishment of estrogen receptor (19, 23). The inhibitory effect of tamoxifen on cellular growth of human mammary cancer cells in culture has also been suggested to be mediated by the competition for the estrogen receptor system, because this effect can be reversed by estradiol (16, 24) even though the role of the estrogen receptor system or estradiol by itself in the process of cellular multiplication *in vitro* is questioned by the ineffectiveness of estrogens to promote cellular growth and DNA polymerase activity (10, 16).

The presence of a specific antiestrogen binding site preferentially localized in estrogen target cells could represent a further step for the investigation of the interactions of these compounds with target tissues; its role in the complex biological responses elicited by antiestrogens remains to be elucidated.

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Distribution of 4-Hydroxy-*N*-desmethyltamoxifen and Other Tamoxifen Metabolites in Human Biological Fluids during Tamoxifen Treatment[1]

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ABSTRACT

Several metabolites of tamoxifen, including 4-hydroxy-*N*-desmethyltamoxifen (metabolite BX), 4-hydroxytamoxifen (metabolite B), *N*-desmethyltamoxifen (metabolite X), the primary alcohol (metabolite Y), and *N*-desdimethyltamoxifen (metabolite Z) were identified and their concentrations determined in fluids and feces from patients receiving chronic tamoxifen treatment. The biological samples investigated were serum, pleural, pericardial and peritoneal effusions, cerebrospinal fluid, saliva, bile, feces, and urine.

In serum, tamoxifen itself, and the metabolites X and Z were the prevailing species, but significant amounts of the metabolites Y, B, and BX were also detected. About 3 h after drug intake tamoxifen as well as Y, B, BX, X, and Z showed a peak in serum. This may be explained by efficient metabolism of the metabolite precursor before being distributed to peripheral compartments. Upon drug withdrawal all metabolites showed first-order elimination curves which paralleled that of tamoxifen suggesting that their rate of elimination exceeded that of tamoxifen and that the serum levels are production rate limited. The protein binding of tamoxifen and its major serum metabolites (Y, X, Z) was determined and found to be higher than 98%. Albumin was the predominant carrier for tamoxifen in human plasma.

The concentrations of tamoxifen and its metabolites in pleural, pericardial, and peritoneal effusions equalled those detected in serum, corresponding to an effusion/serum ratio between 0.2 and 1. Only trace amounts of tamoxifen and metabolite X were detected in cerebrospinal fluid (CSF/serum ratio <0.02). In saliva, concentrations of tamoxifen and X exceeded the amounts of free drug in serum, suggesting active transport or trapping of these compounds in the salivary gland. Bile and urine were rich in the hydroxylated, conjugated metabolites (Y, B, and BX), whereas in feces unconjugated metabolite B and tamoxifen were the predominating species.

INTRODUCTION

The nonsteroid antiestrogen tamoxifen [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene] has since 1973 been widely used for the palliative treatment of breast cancer (1). Tamoxifen has also been evaluated in the management of several other conditions, including pancreatic (2), ovarian and endometrial cancer, anovulatory infertility (3), cyclic mastalgia (4), and trials on prophylactic intervention in women at high risk of developing breast cancer are planned (5, 6).

Tamoxifen seems to be extensively metabolized and knowledge of its biotransformation is important since this is a major determinant of elimination and some metabolites may be active.

Most information on the distribution of tamoxifen and its metabolites are based on animal studies. Borgna and Rochefort found polar metabolites of tamoxifen in plasma, uterus, liver, and oviduct of immature female rats and chickens injected with [^{3}H]tamoxifen. 4-Hydroxytamoxifen and another hydroxylated metabolite, "M2," were the major tritiated metabolites in the cytosol and KCl-extracted nuclear fraction from rat uterus (7). This metabolite may be 4-hydroxy-*N*-desmethyltamoxifen, the formation of which has been demonstrated after incubation with rat liver microsomes (8) and in isolated rat hepatocytes in suspension (9). In the female rat, mouse, monkey, and dog most [^{14}C]tamoxifen was excreted via bile into the feces, but a significant fraction of the biliary radioactivity was reabsorbed and underwent enterohepatic circulation (10). In the rat most tamoxifen metabolites in bile and feces were present as glucuronides and other conjugates (10).

Despite the wide use of tamoxifen as a drug, knowledge on its fate in humans is sparse. Several metabolites of tamoxifen have been identified in human plasma, *i.e.*, the primary alcohol (metabolite Y[3]), metabolite B, metabolite X, metabolite Z (3). Fromson gave a single dose of [^{14}C]tamoxifen to four patients and found that the peak plasma concentration occurred after 4–7 h and the terminal half-life was longer than 7 days (11). Other pharmacokinetic studies confirmed these findings (12). Also in humans, most tamoxifen is excreted as metabolites into feces. Less than 20% are eliminated in the urine (11).

Among the serum metabolites the hydroxylated metabolite B has received particular attention since it has higher *in vitro* affinity for the estrogen receptor than the parent drug (8, 13, 14). Another hydroxylated metabolite, 4-hydroxy-*N*-desmethyltamoxifen (metabolite BX), has similar properties (8).

We recently demonstrated high concentrations of metabolite BX in bile from a patient receiving chronic tamoxifen treatment (15). We here report on the investigation of the presence of metabolite BX and other tamoxifen metabolites in various biological samples from patients treated with tamoxifen. These samples include serum, pleural, pericardial and peritoneal effusions, cerebrospinal fluid, saliva, urine, and bile, and an extract from feces.

MATERIALS AND METHODS

Chemicals. Tamoxifen, metabolite B, and metabolite X were obtained from Pharmachemie B.V., Haarlem, Holland. Metabolite Y, metabolite Z, and metabolite BX were gifts from Imperial Chemical Industries PLC, Pharmaceuticals Div., Macclesfield, Cheshire, UK. The latter reference compound was obtained as a mixture of about 85% *cis* isomer and 15% *trans* isomer. Attempts to enrich the *trans* isomer by boiling in ethanol, were not successful. [*N-methyl*-^{3}H]tamoxifen (specific activity, 84 Ci/mmol) and [^{14}C]tamoxifen citrate S.A. 25 mCi/mmol were obtained from Amersham International, UK. Purity was checked by thin-layer chromatography on silica gel in ethyl ether:triethylamine (99:1). *N*-Methyl-tritiated tamoxifen was highly unstable and was not used in quantitative analysis.

β-Glucuronidase was obtained from Sigma Chemical Company, St. Louis, MO. This β-glucuronidase, which is a preparation of the intestinal juice of the snail, *Helix pomatia*, also contains sulfatase activity

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[3] The abbreviations used are: metabolite Y, *trans*-1(4-β-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene; metabolite B, 4-hydroxytamoxifen; metabolite BX, 4-hydroxy-*N*-desmethyltamoxifen; metabolite X, *N*-desmethyltamoxifen; metabolite Z, *N*-desdimethyltamoxifen; ODS, octadecylsilane; AcN, acetonitrile; LC/MS, liquid chromatography/mass spectrometry; MPA, medroxyprogesteroneacetate; SIM, selected-ion monitoring; CSF, cerebrospinal fluid.

(β-glucuronidase 126,000 units/ml, sulfatase 4000 units/ml).

Monospecific antiserum used for albumin determinations (16) was purchased from Dakopatts, Copenhagen, Denmark.

Patients. 36 patients, who received tamoxifen as a palliative treatment of breast cancer, were included, and they all gave their informed consent to participate in the study. Their age, duration of treatment, dosing, and biological samples obtained, are listed in Table 1.

Sample Collection. Pericardial, pleural and peritoneal effusion, and cerebrospinal fluid were obtained during diagnostic or therapeutic procedures, collected, and stored in glass tubes until analysis. Blood samples were drawn on the same occasions. Bile was collected into polyethylene bags from a biliary T-drain from Patient E. B. with breast cancer and total bile duct occlusion due to metastases.

Blood samples were drawn from three patients (A. B., E. M. C., and G. T.) after tamoxifen was discontinued to obtain the decay curves for tamoxifen and its metabolites. Blood samples were collected at intervals of 4 to 14 days for 35 to 41 days after drug withdrawal. Feces were collected from three patients (A. H., K. R., and A. L. H.) for 3 days and urine from 13 patients for 24 h. Urine was collected into plastic cans. Saliva was obtained from 11 patients while chewing waxed sheet (Parafilm) to stimulate salivary flow.

Blood samples were obtained in these patients at the time of collecting feces, urine, or saliva.

All samples were stored in darkness at −20°C until analysis.

Sample Processing. The bile and urine were thawed, and treated with β-glucuronidase from *Helix pomatia*, essentially as described by Bakke and Scheline (17). To increase the yield of unconjugated tamoxifen and metabolites from urine, the treatment with β-glucuronidase was extended to 24 h, and the amount of β-glucuronidase used was increased from 2520 to 5040 units/ml urine. The yield was also increased by resuspending the urine precipitate by vigorous shaking before the β-glucuronidase-treatment. After incubation with the enzyme, the pH was adjusted to 7 by adding 1 N NaOH. The neutralized samples were mixed with an equal volume of AcN, and after centrifugation the supernatants were transferred to sample vials, capped, and analyzed.

In a preliminary study aliquots of urine (5 ml) were refluxed for 2.5 h with water (5 ml) and titrated to pH 1 with 18 N sulfuric acid. The samples were then neutralized and handled as described above.

Feces were mechanically homogenized, and about 3 g of feces were then mixed with 20 ml of water or 50% AcN, vigorously shaken and treated with ultrasound for 5 min. The extract was then centrifuged for 15 min at 420 × g_{av}. The supernatant obtained from extraction with water was treated with β-glucuronidase as described in the previous paragraph for urine. The solution obtained by direct AcN extraction was properly diluted with mobile phase, transferred to sample vials, capped, and analyzed.

The samples of pleural, pericardial and peritoneal effusions, cerebrospinal liquor, and saliva were treated with AcN, as previously described for serum (18).

Determination of Protein Binding. Protein binding of tamoxifen was determined both for drugs in patient serum by ultracentrifugation and for radiolabeled drug added to drug free serum by column chromatography and equilibrium dialysis.

Serum (5 ml) from Patient B. L. who had been treated with 40 mg tamoxifen b.i.d. for 78 days, was transferred to Ultra-Clear tubes from Beckman and centrifuged at 215,000 × g_{av} for 24 h in an ultracentrifuge model Beckman L8-60M. The temperature was kept at 4°C. The centrifugation tubes were harvested by careful aspiration of fractions of 400 μl from the top of the generated gradient. The concentrations of tamoxifen and its metabolites and albumin were determined in the separate fractions and in the same serum sample before centrifugation.

[^{3}H]Tamoxifen (3 μCi) was added to serum diluted 1:1 with isotonic phosphate buffer (pH 7.4), and incubated for 24 h at 4°C. A sample of 1 ml was subjected to affinity chromatography on concanavalin A-Sepharose 4B from Pharmacia, as described previously (19). Another sample was analyzed by gel filtration on Sephadex G-100 column (2.5 x 70 cm). Radioactivity was determined in 0.5-ml aliquots of eluent fractions. Absorbance was determined at 280 nm.

Equilibrium saturation analysis was performed by dialysis of five different concentrations of [^{14}C]tamoxifen citrate against a 60-μM solution of human serum albumin (Koch-Light Chemicals, UK) in isotonic phosphate buffer, pH 7.4 (20).

High-Performance Liquid Chromatography. We used a liquid chromatography system which was developed for the determination of tamoxifen and metabolites in serum (18). The assay was modified to improve the separation and isolation of the early eluting, hydrophilic metabolites. The method and the modification are briefly described below.

Large samples of 250 μl were injected into a small precolumn with an internal diameter of 0.21 cm, packed with 5 μm ODS material. The length of this column was increased from 2 to 3 cm (15). The samples were on-column concentrated by equilibrating the precolumn with 50% AcN in water, containing 3 mM acetic acid and 2 mM diethylamine. The analytes were then directed into an analytical ODS-Hypersil column (0.21 x 10 cm) by changing the mobile phase followed by column switching. The composition of the mobile phase was 91% AcN containing 1 mM acetic acid and 0.67 mM diethylamine, and the flow rate was 0.3 ml/min. Small adjustments in the AcN concentration of the mobile phase were made to compensate for different composition of the extracts.

Tamoxifen and its metabolites were eluted in the following order: metabolite Y, metabolite B, metabolite BX, tamoxifen, metabolite Z, and metabolite X. These compounds were postcolumn converted to fluorophors by UV illumination while passing through a quartz tube, and then monitored by a fluorescence detector.

Table 1 *Patient characteristics*

Patient	Age	Treatment: Duration (days)	Treatment: Dose	Biological sample[a]
UA	71	77	30 mg daily	Pleural effusion
		203	30 mg daily	Saliva
AB	65	56	30 mg daily	Multiple serum samples[b]
EB	57	71	30 mg daily	Bile[c]
SB	70	184	30 mg daily	Saliva
EMC	61	118	30 mg daily	Multiple serum samples[b]
MC	70	23	30 mg daily	Pericardial effusion
		112	30 mg daily	Pericardial effusion
DE	64	720	30 mg daily	Urine
IE	76	73	30 mg daily	Multiple serum samples,[d] urine
		134	30 mg daily	Multiple serum samples,[d] urine
SE	73	161	30 mg daily	Saliva
AF	67	20	30 mg daily	Pleural effusion
EF	65	21	30 mg daily	Peritoneal effusion
AH	62	57	30 mg daily	Feces
ALH	58	13	30 mg daily	Feces
RH	73	11	30 mg daily	Pleural effusion
		66	30 mg daily	Pleural effusion
		93	30 mg daily	Pleural effusion
		178	30 mg daily	Saliva
ITH	58	175	30 mg daily	Urine
EJ	51	14	30 mg daily	Pleural effusion
DK	51	22	30 mg daily	Pleura
BL	50	835	30 mg daily	Cerebrospinal fluid
		78[e]	40 mg b.i.d.	
OL	83	716[e]	40 mg b.i.d.	Urine
BO	70	120	30 mg daily	Urine
JGO	64	172	30 mg daily	Saliva
EN	67	28	30 mg daily	Saliva
SP	72	651	30 mg daily	Saliva
AR	42	10	30 mg daily	Pleural effusion
ALR	68	135	30 mg daily	Urine
KR	59	47	30 mg daily	Feces, Urine
		54	30 mg daily	Urine
NS	68	62[e]	40 mg b.i.d.	Pleural effusion
OS	79	296	30 mg daily	Urine
SBS	62	296	30 mg daily	Urine
RS	79	132	30 mg daily	Saliva
AT	76	68	30 mg daily	Urine
		129	30 mg daily	Multiple serum samples,[d] urine
GT	74	990	30 mg daily	Multiple serum samples[b]
ST	72	54	30 mg daily	Saliva
KZ	81	133	30 mg daily	Saliva
LMØ	41	480	30 mg daily	Saliva
MØ	44	48	30 mg daily	Urine

[a] Refers to biological sample other than a single serum sample, which was obtained from all patients.
[b] After drug withdrawal.
[c] External biliary catheter.
[d] During one dosing interval.
[e] Days after dose escalation from steady state on 30 mg daily.

The instruments and the construction of the postcolumn converter have been described (18).

Liquid Chromatography/Mass Spectrometry. The analytical column was connected to a LC/MS, thermospray system (Model 201; Vestec, Houston, TX). Before entering the thermospray, the effluent from the column was mixed with 0.1 M ammonium acetate reagent, delivered at a rate of 0.7 ml/min via a zero dead volume T-connector.

Determination of Albumin. Albumin was quantitated in individual fractions by "rocket" immunoelectrophoresis (16). Column dimensions were 2.5 × 70 cm and flow rate 52 ml/h. Temperature, 4°C.

RESULTS

Identification of Tamoxifen and Its Metabolites in Biological Fluids and Extracts. Samples of serum, pericardial, pleural and peritoneal effusions were deproteinized with AcN and analyzed by microdispersion LC and fluorescence detection, as described in a previous publication (18). Tamoxifen, metabolites Y, X, and Z, could be demonstrated in the chromatogram in substantial amounts whereas metabolites BX and B were present only in low concentrations (Fig. 1). The metabolites seem to be unconjugated in these fluids, since β-glucuronidase treatment did not increase peak height (data not shown).

The amounts of metabolite BX in serum were below the detection limit of our LC/MS system. To verify the identity of this metabolite it was concentrated by extraction of 10 ml serum with hexane-2% butanol, evaporated and redissolved in 500 μl 50% AcN. A compound with molecular ion $(M + 1)^+$ of 374 *m/z*, corresponding to that of authentic metabolite BX, cochromatographed with the fluorescence peak of metabolite BX (data not shown).

The amount of tamoxifen and metabolites in cerebrospinal fluid was below the detection limit of our routine assay based on fluorescence detection (Fig. 1).

In urine and bile the existence of tamoxifen, metabolite BX as well as other hydroxylated metabolites like metabolites Y and B could be verified with LC/MS (SIM). This is shown for metabolite BX in Fig. 2. In feces the amount of metabolite BX was small and the abundance of the 374^+ ion gave a response only slightly but significantly above the baseline (Fig. 2). Metabolite B and tamoxifen could be detected and verified in feces by this method.

Fig. 1. Chromatograms of serum and various biological fluids. Samples from *A*, serum; *B*, pleural effusion; *C*, pericardial effusion; *D*, peritoneal effusion; and *E*, cerebrospinal fluid were subjected to reversed-phase chromatography, as described in the text. The samples are from different patients.

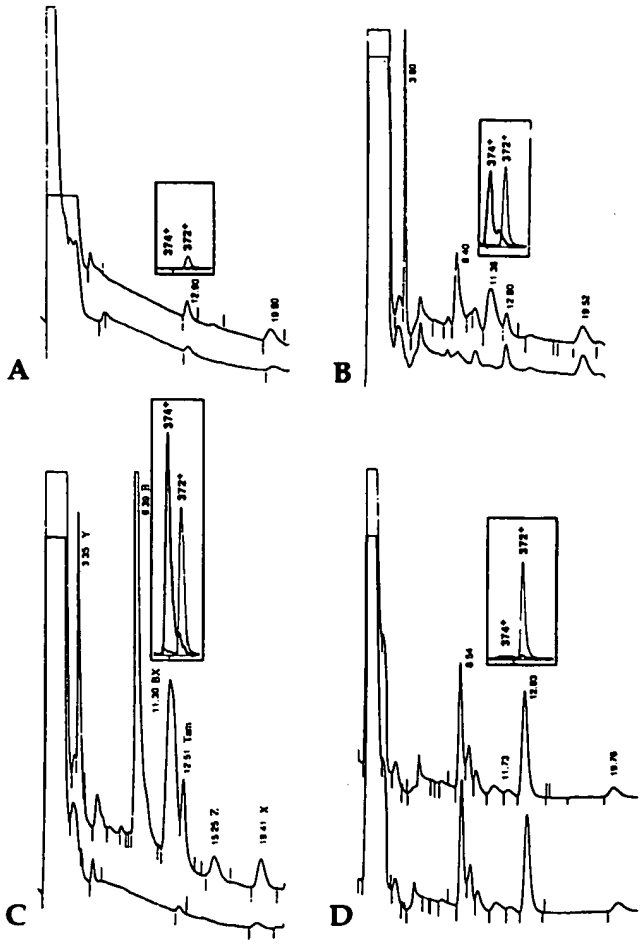

Fig. 2. Chromatography of extracts from *A*, saliva; *B*, urine; *C*, bile; and *D*, feces; and identification of compounds with LC/MS. The samples were divided into two portions, one of which was treated with β-glucuronidase. Samples were subjected to reversed-phase chromatography as described in the text. The fluorescence profiles were obtained after postcolumn photoactivation (18). *Low traces*, untreated samples; *upper traces*, samples treated with β-glucuronidase. *Insets*, LC-SIM traces for the M^+ ion for tamoxifen (374 *m/z*) and metabolite BX (372 *m/z*). In these separate runs the postcolumn reactor was by-passed.

Metabolite Concentrations and Kinetics during Chronic Dosing. Fig. 3 shows the variations in serum concentrations of tamoxifen and its metabolites, including metabolite BX, throughout 24 h in two patients (A. T., I. E.) during steady state (Table 1). About 2 h after drug ingestion, serum tamoxifen showed a distinct peak, which was followed by a less pronounced serum peak. The concentration then slightly declined. Similar serum profiles were observed for metabolites Y, B, BX, X, and Z (Fig. 3).

The serum concentration curves were determined for tamoxifen and its metabolites in three patients after drug withdrawal. The elimination curves were monophasic and corresponded to serum half-lives between 7 and 11 days for tamoxifen and 10 and 11 for metabolite X. Notably, the curves for metabolites Y, B, BX, X, and Z were parallel to that of the parent compound, tamoxifen (Fig. 4).

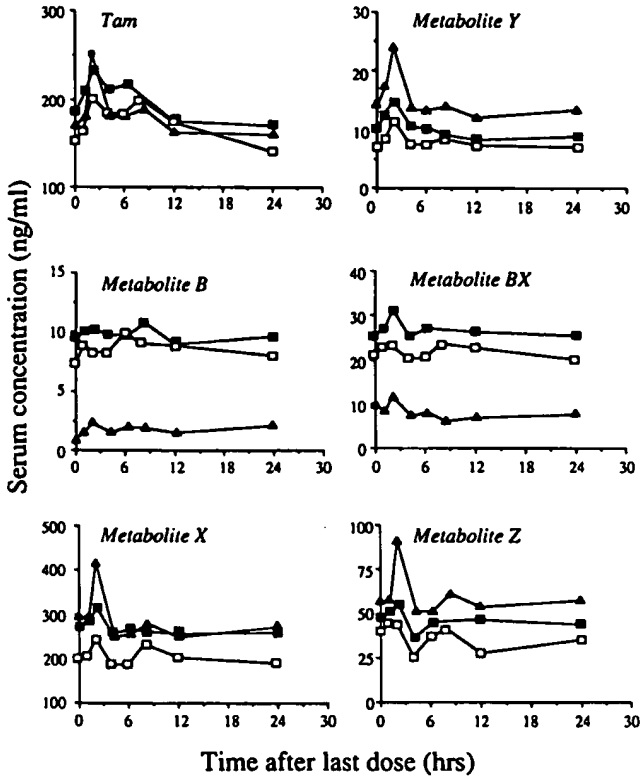

Fig. 3. Fluctuations in serum levels of tamoxifen and metabolites during one dosing interval. 30 mg tamoxifen was given at time 0. ▲, Patient AT; □, Patient IE (8/8/87); ■, Patient IE (10/9/87).

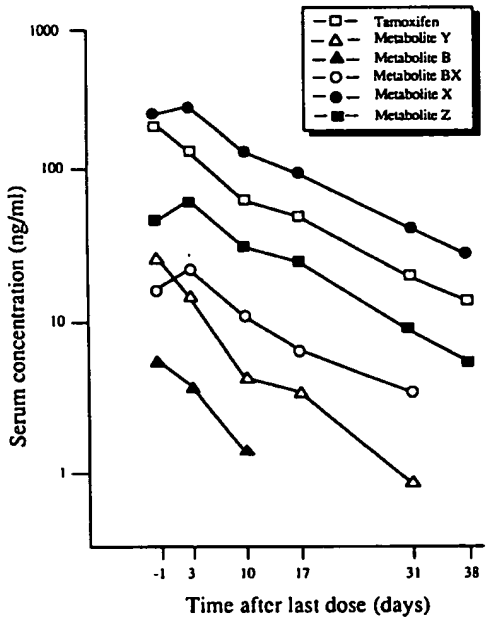

Fig. 4. Elimination curves for tamoxifen and metabolites in serum after tamoxifen withdrawal. Patient (*A. B.*) was treated with 30 mg tamoxifen daily for 60 days. At time 0 days tamoxifen was replaced with MPA.

The apparent terminal distribution volume (V_z) for tamoxifen during steady state could be calculated from the equation (21):

$$V_z = \frac{D \cdot \tau}{C \cdot k_{el}}$$

where τ is the dosing interval, C the mean concentration during steady state, and k_{el} the elimination constant.

V_z values were 52 liter/kg (A. B.), 61 liter/kg (G. T.) and 53 liter/kg (E. M. C.). The Patient A. B. changed directly over from tamoxifen to MPA after tamoxifen treatment for 8 weeks. Patient E. M. C. used acetaminophen, allopurinol, codeine, digoxin, furosemide, nifedipine, and warfarin, whereas G. T. used no other drugs.

Protein Binding. Serum from a patient (B. L.) receiving 40 mg tamoxifen b.i.d. was subjected to ultracentrifugation. A gradient of albumin was created, and amount of albumin in the top fraction was 0.5% of that in whole serum. The concentration of tamoxifen, metabolites Y, X, and Z closely followed the albumin concentration (data not shown). Based on the residual amount of albumin in the top fraction and the detection limit of the method (about 1 ng/ml) the protein binding of these three compounds were calculated to be higher than 98%. Because of the low level of metabolites B and BX in serum, the protein binding of these compounds could not be assessed.

The protein binding was further studied by characterizing the interaction of labelled drug with serum proteins. Most [^{3}H] tamoxifen added to serum comigrated with albumin when fractionated by gel filtration on Sephadex G 100. A peak of similar magnitude and elution volume was observed when serum was replaced by pure human serum albumin at a concentration equivalent to that present in serum (Fig. 5). Furthermore, Scatchard analysis (22) (not shown) of the binding data obtained from equilibrium dialysis of [^{14}C]tamoxifen *versus* human serum albumin revealed an association constant of 1.3×10^5/M and approximately 1 binding site per molecule.

From these data one can calculate that albumin alone could bind 98.8% of tamoxifen in serum. Other binding proteins do not seem to be involved to any great extent. Fractionation of serum incubated with labeled tamoxifen on concanavalin A-Sepharose columns (19) showed no label to be associated with the adsorbed glycoprotein fraction (Fig. 5). This excludes significant binding to α_1-acid glycoprotein and the steroid-binding proteins cortisol binding globulin and sex hormone binding globulin.

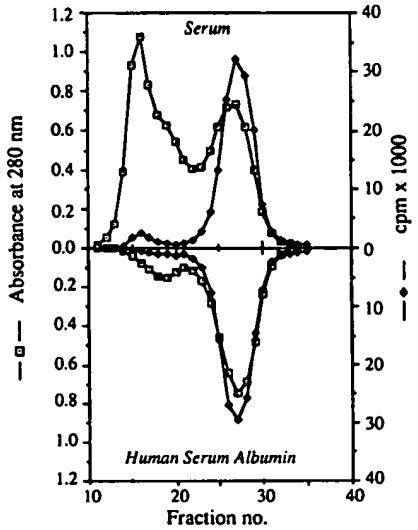

Fig. 5. Gel filtration of human serum and human serum albumin incubated with [^{3}H]tamoxifen. *Top*, gel filtration on Sephadex G-100 (2.5 x 70 cm) of 1 ml serum diluted 1:1 with phosphate buffer (pH 7.4) incubated with 3 μCi [^{3}H] tamoxifen. Flow rate was 52 ml/h, temperature 4°C. Radioactivity was determined in 0.5-ml aliquots of eluent fractions. Absorbance was measured at 280 nm. Albumin was quantitated in each fraction by "rocket" immunoelectrophoresis. *Bottom*, gel filtration as in the top, but this time 2 ml 2% human serum albumin was used instead of human serum.

A small fraction of labeled tamoxifen was associated with large protein(s) eluted in the void volume of Sephadex G-100. This binding could be due to the presence of a high affinity, low capacity binding protein analogous to the antiestrogen binding site on rat serum low density lipoprotein (23).

Distribution into Pleural, Pericardial and Peritoneal Effusions and Cerebrospinal Fluid. The concentrations of tamoxifen and its serum metabolites were determined in pleural, pericardial and peritoneal effusions from nine patients receiving chronic tamoxifen dosing. A serum sample was drawn and analyzed on the same occasions (Table 2).

There was only a marginal increase (<5%) in concentrations of tamoxifen and its metabolites in these effusions as well as serum after incubation with β-glucuronidase (data not shown), suggesting that they exist mainly as unconjugated species in these fluids.

The distribution ratios between these fluids and serum varied between 0.3 and 1 in most cases (Table 2). These values were compared with the distribution of albumin (most ratios between 0.5–0.8) but no obvious correlation could be demonstrated. The distribution ratios for the different compounds were similar. Tamoxifen and its metabolites therefore seem to be distributed into these fluids to about the same extent. Notably, the distribution of tamoxifen and its metabolites showed a marked inter patient variation (Table 2). For example, the ratio was between 0.2 and 0.6 for Patient R. H. on three different occasions (on Days 11, 66, and 93 after initiation of treatment) and between 0.7 and 1.2 for Patient M. C. on two occasions (on Days 23 and 112).

The amounts of tamoxifen and its metabolites in cerebrospinal liquor from Patient B. L. were below the detection limit (about 1 ng/ml) of our routine LC method. We improved the sensitivity of the method by concentrating these compounds from 1.5 ml of CSF on top of the guard column. On-column concentration was improved by equilibrating the system with water, and then eluting it as described in "Materials and Methods." Tamoxifen and metabolite X was then detected in significant, but low, concentrations. The CSF/serum ratio was of the same magnitude for tamoxifen and metabolite X as for albumin (Table 2).

Tamoxifen and Metabolite X in Saliva. Analysis of saliva from 11 patients showed that the compounds most abundant in serum, tamoxifen, and metabolite X were present in substantial amounts, whereas the other tamoxifen metabolites were essen-

Table 2 *Distribution of tamoxifen and its metabolites between serum and pleural, pericardial and peritoneal effusions and between serum and CSF*

Patient	Days of treatment	Sample	TAM[a] (ng/ml)	Y[a] (ng/ml)	B[a] (ng/ml)	BX[a] (ng/ml)	X[a] (ng/ml)	Z[a] (ng/ml)	Albumin (g/liter)
UA	71	Pleural effusion	87.1	34.8	2.0	7.2	176.2	50.6	28
		Serum	173.0	67.1	4.3	8.0	278.8	66.6	40
		Ratio	0.50	0.52	0.47	0.90	0.63	0.76	0.70
AF	20	Pleural effusion	148.1	3.1	3.0	2.8	114.9	9.6	15
		Serum	321.7	8.5	7.1	7.8	200.9	20.8	25
		Ratio	0.46	0.37	0.42	0.36	0.57	0.46	0.60
RH	11	Pleural effusion	63.7	3.7	0.8	1.3	56.9	8.3	30
		Serum	145.2	13.3	3.3	4.0	121.0	18.3	38
		Ratio	0.44	0.28	0.24	0.33	0.47	0.45	0.79
RH	66	Pleural effusion	72.0	8.0	1.5	5.5	121.3	29.3	30
		Serum	151.5	28.3	3.5	8.6	203.6	47.5	34
		Ratio	0.48	0.28	0.43	0.64	0.60	0.62	0.88
RH	93	Pleural effusion	73.7	9.1	0.8	3.4	110.3	26.3	25
		Serum	198.0	33.9	4.4	13.4	281.0	65.2	38
		Ratio	0.37	0.27	0.18	0.25	0.39	0.40	0.66
EJ	14	Pleural effusion	66.4	9.2	2.0	9.2	85.4	23.1	34
		Serum	95.5	13.1	5.5	10.0	103.2	25.8	47
		Ratio	0.70	0.70	0.36	0.92	0.83	0.90	0.72
DK	22	Pleural effusion	50.5	4.7	0.3	2.2	113.0	15.8	26
		Serum	138.7	13.2	0.7	3.6	187.3	48.0	40
		Ratio	0.36	0.36	0.43	0.61	0.60	0.33	0.65
AR	10	Pleural effusion	67.5	1.7	1.2	0	38.9	0	16
		Serum	159.7	5.2	4.5	3.0	75.7	3	25
		Ratio	0.42	0.33	0.27		0.51		0.64
NS	62[b]	Pleural effusion	200.0	43.1	3.5	3.5	395.7	86.8	30
		Serum	240.2	57.7	3.8	3.9	410.1	89.8	40
		Ratio	0.83	0.75	0.92	0.90	0.97	0.97	0.75
MC	23	Pericardial effusion	96.0	15.9	3.0	13.4	152.1	25.7	24
		Serum	89.6	13.5	3.1	15.4	128.0	26.2	25
		Ratio	1.07	1.18	0.97	0.87	1.19	0.98	0.96
MC	112	Pericardial effusion	71.4	19.2	2.5	10.5	149.0	39.3	24
		Serum	79.0	22.5	3.5	10.0	163.5	42.1	28
		Ratio	0.90	0.85	0.71	1.05	0.91	0.93	0.86
EF	21	Peritoneal effusion	179.9	15.2	4.0	9.6	101.1	17.9	13
		Serum	227.9	23.7	7.0	28.0	238.6	54.9	25
		Ratio	0.79	0.64	0.57	0.34	0.42	0.33	0.69
BL	835	CSF	1.1				1.2		0.2
		Serum	103.2	15.8	1.6	6.7	171.5	33.7	31.8
		Ratio	0.011				0.007		0.006

[a] TAM, tamoxifen; Y, metabolite Y; B, metabolite B; BX, metabolite BX; X, metabolite X; Z, metabolite Z.
[b] 62 days after dose escalation from 30 mg daily to 40 mg twice daily.

tially absent. Furthermore, we found no strict correlation between serum level and the concentration in saliva (saliva/serum ratio between 0.003 and 0.081 for tamoxifen and 0.002 and 0.040 for metabolite X), but saliva from patients with high serum levels often contained relatively high amounts of tamoxifen and metabolite X (Table 3). Notably, there was a correlation between the amount of tamoxifen and metabolite X in saliva (Fig. 6), suggesting that salivary tamoxifen is not derived from tablet residues.

Table 3 *Distribution of tamoxifen and metabolite X into saliva*

Patient	Sample	Tamoxifen (ng/ml)	Metabolite X (ng/ml)
UA	Saliva	0.6	0.8
	Serum	110.6	202.8
	Ratio	0.005	0.004
SB	Saliva	1.2	1.3
	Serum	146.0	197.3
	Ratio	0.008	0.007
SE	Saliva	7.8	5.8
	Serum	283.5	215.0
	Ratio	0.028	0.027
RH	Saliva	2.2	3.0
	Serum	124.6	186.8
	Ratio	0.018	0.016
EN	Saliva	0.6	0.7
	Serum	104.4	105.9
	Ratio	0.006	0.007
JGO	Saliva	2.1	1.2
	Serum	188.5	206.8
	Ratio	0.011	0.006
SP	Saliva	1.7	1.1
	Serum	90.3	178.3
	Ratio	0.019	0.006
RS	Saliva	0.3	1.1
	Serum	23.4	69.9
	Ratio	0.013	0.016
TS	Saliva	0.6	0.8
	Serum	192.0	368.8
	Ratio	0.003	0.002
KZ	Saliva	5.0	4.8
	Serum	252.4	425.8
	Ratio	0.020	0.011
LMØ	Saliva	14.6	11.1
	Serum	179.0	279.6
	Ratio	0.081	0.040

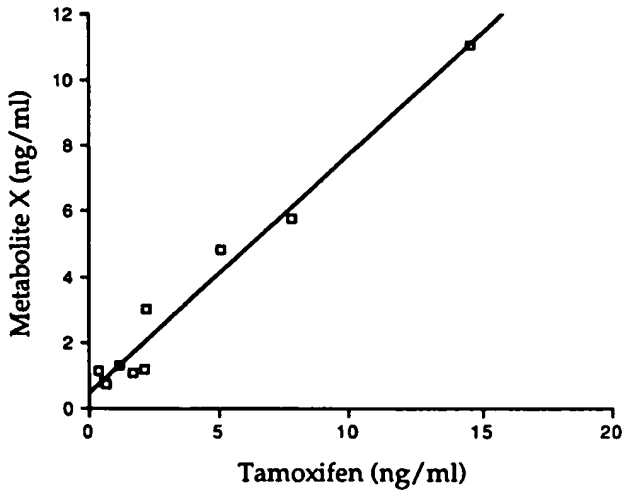

Fig. 6. Correlation between tamoxifen and metabolite X in saliva.

Urine, Bile, and Feces. Fig. 2 shows that the hydroxylated metabolites Y, B, and BX, are the predominating species in urine as well as in bile. Metabolite B and the new metabolite, BX, are about equally abundant in urine, whereas the amount of metabolite BX exceeds that of metabolite B in bile. These metabolites are probably conjugated in urine and bile since their chromatographic peaks appear after β-glucuronidase treatment (Fig. 2, *B* and *C*).

The bile serum ratio for Patient E. B. after 71 days of tamoxifen treatment and 21 days external T-tube was for tamoxifen and metabolites Y, B, BX, X, and Z 0.3, 1.3, 69.0, 47.0, 0.01, and 0.2, respectively.

In feces the metabolite profile is quite different (Fig. 2*D*). Large amounts of tamoxifen and metabolite B were found, whereas smaller amounts of metabolite BX and X were recovered. The recovery of these compounds was about twofold higher when feces was extracted with AcN compared with water (data not shown). Treatment of the fecal extract from one patient with β-glucuronidase increased concentration of tamoxifen and metabolites by less than 27%, whereas the chromatographic profile was unchanged after β-glucuronidase treatment in the two other samples investigated. This suggests that tamoxifen and metabolites are mainly unconjugated in feces.

The amounts of tamoxifen and its metabolites found in urine, bile and feces after β-glucuronidase treatment are summarized in Table 4.

In a preliminary investigation we have found that the amounts recovered from urine after boiling with sulfuric acid increased more than 10-fold. This indicates that other conjugates than glucuronides may be present. However acid hydrolyses may alter the aglycone. These results are therefore difficult to interpret.

DISCUSSION

The present paper describes the distribution of tamoxifen and its metabolites into effusions, cerebrospinal fluid, saliva, urine, bile and feces of patients receiving chronic dosing with tamoxifen. The metabolites investigated include metabolite BX which was recently discovered in our laboratory to be present in human bile (15), but evaluation of its role will depend on knowledge of its concentration in serum and other fluids. Notably, this compound exists in serum and effusions in small amounts which exceed that of metabolite B (Fig. 1, Table 2). Thus, these hydroxylated metabolites may reach the target cells. This may be important since they have a higher affinity than tamoxifen towards the estrogen receptor and may therefore possess significant biological activity (8). However, the largest amounts of metabolite BX are found in conjugated form in excretory fluids like bile and urine (Fig. 2, Table 4), suggesting that metabolism along this pathway contributes to the metabolic clearance of tamoxifen.

Table 4 *Tamoxifen and metabolites extracted from 24-h samples of urine, bile and feces*

All samples were treated with β-glucuronidase, and the values are given in μg/24 h.

Material	TAM	Metabolite Y	B	BX	X	Z
Urine[a]	3.5 ± 2.2	5.6 ± 4.3	8.9 ± 5.1	11.4 ± 5.2	4.7 ± 3.0	0
Bile[b]	12	5	68	98	1	2
Feces[c]	609	24	314	141	92	2
	230–1092	13–41	123–579	92–189	61–121	0–7

[a] Values are given as mean ± SD, $n = 14$.
[b] Sample from one single Patient (E. B.) with liver metastases and external biliary drainage.
[c] Values are given as mean and range, $n = 3$.

We monitored serum tamoxifen, metabolite BX and other metabolites during one dosing interval in two patients (Fig. 3). The concentrations are within the range reported by others (24). Tamoxifen in serum had a peak concentration which subsided within 5 h after drug intake (Fig. 3). This transient increase is probably related to drug absorption (25). A second moderate increase in serum concentration could be explained by enterohepatic circulation which has been reported for tamoxifen in the rat and dog (10).

Most metabolites, especially Y, X, and Z, showed a serum peak concurrent with the absorption peak for tamoxifen (Fig. 3). This could be explained by efficient formation of these metabolites from tamoxifen before the parent drug is distributed to peripheral compartments.

The elimination curve for tamoxifen after drug withdrawal was monoexponential and consistent with a half-life of 7–11 days (Fig. 4) which is in accordance with data reported by others (12). In some patients (A. B., E. M. C., and G. T.) the curves of metabolite X and Z paralleled the tamoxifen decay curve. The same relation to tamoxifen seems to exist for metabolite Y, B, and BX, but determination of the terminal data points was hampered by low concentrations approaching the detection limit of the assay. Our data suggest that the serum levels of metabolites X and Z and probably of Y, B, and BX are production rate limited, and that the elimination rates for these metabolites equal or exceed that of tamoxifen. This conclusion is in variance with the results from single dose experiments showing that metabolite X has a longer half-life than tamoxifen (12, 26). Our data should be interpreted with caution because our patients have various manifestations of breast cancer. Furthermore, one of them changed over from tamoxifen to MPA, a drug which may influence tamoxifen pharmacokinetics (26). Another used a combination of eight drugs including warfarin. The possibility that tamoxifen (and its metabolites) may affect its own metabolism through influence on microsomal enzymes (27) should also be considered.

To the best of our knowledge the distribution volume of tamoxifen has not been previously determined. We calculated the terminal distribution volume (V_z) for tamoxifen to be 50–60 liter/kg. This estimation is based on the assumption that the bioavailability (F) of tamoxifen is equal to one, as reported in animals (10). F has not been directly determined in humans for this drug because no intravenous formulation of tamoxifen is available. However, Adam *et al.* (12) demonstrated equal availability of different oral formulations of tamoxifen, suggesting efficient absorption.

The protein binding of tamoxifen and its metabolites in plasma may affect distribution as well as elimination, but data on protein binding of tamoxifen are sparse (23, 28, 29) and have not been published for tamoxifen metabolites. We investigated the protein binding by ultracentrifugation of patient serum and found that tamoxifen and metabolites Y, X, and Z cosedimented with serum albumin. Our data were consistent with protein binding higher than 98% for these compounds during chronic tamoxifen administration.

The gel filtration experiment with labeled tamoxifen (Fig. 5) suggests that albumin is the main but not the sole carrier of tamoxifen in human serum. A small fraction of labeled tamoxifen was associated with large protein(s) eluting in the void volume of the Sephadex G-100 column (Fig. 5). This binding could be due to the presence of a high affinity, low capacity binding protein analogous to the tamoxifen binding site on low density lipoprotein described in rat serum (23). In view of the high concentration of tamoxifen, *i.e.*, micromolar range, circulating in the blood of treated patients, the importance of such a binding protein seems to be negligible compared to albumin, even at the very low (nM) concentration of tamoxifen investigated (Fig. 5).

The equilibrium dialysis studies revealed high affinity binding of tamoxifen to serum albumin, and there is about one site per albumin molecule. From these parameters and the albumin concentration in human serum one can calculate that albumin alone may bind 98.8% of the tamoxifen in serum. However, the binding parameters obtained for tamoxifen should be viewed with some caution. Only 60% of radiolabeled tamoxifen was recovered in our dialysis experiments (data not shown). We have observed that free tamoxifen adheres to glass surfaces and membranes during dialysis as well as ultracentrifugation. This may lead to underestimation of the free tamoxifen fraction.

Fractionation of serum incubated with labeled tamoxifen on a concanavalin A-Sepharose affinity column showed no radioactivity to be associated with the absorbed glycoprotein fraction (data not shown). This excludes tamoxifen binding to steroid-binding proteins like cortisol binding globulin and sex hormone binding globulin, as well as to α_1-acid-glycoprotein (19). This finding is important because α_1-acid-glycoprotein is a highly fluctuating "acute phase protein" which increases during malignant diseases (30). α_1-Acid-glycoprotein accounts for significant protein binding of many lipophilic basic drugs (30) and has also been shown to bind to steroids (31).

The high degree of binding of tamoxifen to human serum albumin is a factor which tends to retain the drug within the circulation. This is in contrast to the relatively large volume of distribution for tamoxifen (V_z about 50–60 liter/kg), which is in accordance with recovery of high concentration of radioactive tamoxifen in several tissues following i.v. injection into mice (32) and rats (33). Due to the limited binding capacity of the estrogen receptor this may only contribute to the binding of a small fraction of tamoxifen in tissue. However, there are high affinity, high capacity binding sites for tamoxifen, so-called "antiestrogen binding sites," in most tissues (34–36). Such peripheral sites may contribute to the extensive distribution and long half life of tamoxifen. In addition, tamoxifen is a lipophilic drug which may accumulate in brain (32) and fat tissue.

Tamoxifen and all serum metabolites are readily distributed into peritoneal, pericardial, and pleural effusions (Table 2) where these compounds may affect cancer cells. Others have reported on the attainment of a rapid equilibrium of drugs between serum and such effusions (37) which are regarded as shallow compartments. The differences in concentrations between serum and these effusions may be related to fluctuation in serum levels during a dosing interval (Fig. 3) and the turnover of these fluids.

Only trace amounts of tamoxifen and metabolite X (about 0.5% of serum level) were found in cerebrospinal fluid from one patient receiving 30 mg tamoxifen daily (Fig. 1, Table 2). To detect these small concentrations we had to add on-column concentration of extract of 1.5 ml cerebrospinal fluid to our routine assay. Others have reported no detectable tamoxifen in cerebrospinal fluid from a patient receiving 150 mg tamoxifen twice daily (38).

Our results show that tamoxifen and metabolite X cross the blood-brain barrier. There are several possible reasons why only small amounts were detected. Free tamoxifen and metabolites may be avidly adsorbed from a solution like CSF with low protein content to surfaces during sample collection. Assuming that protein-binding of tamoxifen and metabolites approaches 100% in CSF, the protein-bound fraction accounts for most of the drug also in this fluid. Thus, low albumin content explains

the low drug concentration in CSF. The observation that the CSF/serum distribution ratios for tamoxifen, metabolite X, and albumin are nearly equal (Table 2) supports this possibility.

The presence of protein-bound tamoxifen in CSF implies that at least trace amounts of free, active drug exist. Free drug may become distributed into tumor and brain tissue. Notably, Wilking (32) found eight times higher concentrations of radioactivity in brain tissue then in blood of mice injected with [^{14}C] tamoxifen. Small amounts of drug may become available to brain metastases. Such cancer cells are often estrogen receptor negative (39). This may explain the lack of effect of tamoxifen on brain metastases (39).

Compared with cerebrospinal fluid, saliva was rich in the two compounds most abundant in serum, tamoxifen, and metabolite X. Trace amounts of metabolite Z were also found in some samples (Fig. 1). No strict correlation with serum levels was found (Table 3). This could be related to partial drug and metabolite adsorption, which could be demonstrated to occur to waxed sheet (Parafilm) chewed by patients to stimulate salivary flow.

Comparing the protein-binding of tamoxifen and metabolite X (higher than 98%) with the distribution of these compounds into saliva (distribution ratio of 0.004–0.081, Table 3) it is suggested that the level of drug in saliva exceeds the concentration of free drug in serum. High concentrations in saliva lends support to the conclusion that lipid soluble drugs readily cross the gland epithelium (40). For some antiepileptic drugs levels in saliva have been regarded to reflect the free fraction (41). At least two explanations could be offered for the disparity between the unbound fraction and the salivary levels. Firstly, tamoxifen and its predominating serum metabolites may be actively transported into the saliva or trapped in the saliva through interaction with salivary proteins including albumin (42), or because of pH-dependent change in drug ionization (43). Secondly, the free fraction of tamoxifen and tamoxifen metabolites in serum is in fact underestimated due to adsorption, as discussed above.

High concentrations of tamoxifen and tamoxifen metabolites in the salivary glands point to the possibility that these compounds may be accumulated in other exocrine glands as well. In mice injected with [^{14}C]tamoxifen, the drug was enriched in pancreas and lung (32). Tamoxifen is presently under evaluation as a drug in the treatment of pancreas cancer (2).

Conjugated, hydroxylated tamoxifen metabolites (Y, B, and BX) seem to prevail in bile and urine (Fig. 2, Table 4) suggesting that biotransformation to such species are important processes in tamoxifen elimination. In addition, conjugated tamoxifen could also be recovered in significant amounts from bile (Fig. 2, Table 4). However, our results with bile should be interpreted with caution since they are obtained from a single patient with liver metastases and bile drainage; both are factors which may modify bile composition (44). The high bile/blood concentration ratio of metabolites B and BX suggests an active secretory process responsible for their transfer from blood to bile (45). From bile these compounds reach feces which seems to be a major excretory route for tamoxifen in humans (11).

The metabolite profile in feces differs markedly from that of bile and urine. Tamoxifen and metabolites exist in feces mainly as unconjugated species. This may be explained by deconjugation catalyzed by enzymes derived from intestinal microorganisms (46).

Tamoxifen itself and metabolite B are the most abundant compounds in feces. Significant amounts of metabolite BX are also detected (Fig. 2, Table 4). Thus, these hydroxylated metabolites may represent quantitatively important excretory products of tamoxifen, as has been suggested for metabolite B in laboratory animals and humans by others (10, 11). It is possible that tamoxifen in feces is produced through the action of bacterial enzymes on tamoxifen metabolite(s). This is a prerequisite for the proposed (10) enterohepatic circulation of tamoxifen.

Metabolite Y was recovered from feces in only trace amounts (Fig. 2, Table 4) but this metabolite is abundant in bile (Fig. 2) and present in significant amounts in serum from patients receiving high-dose tamoxifen (38). These findings are consistent with reabsorption of metabolite Y from the intestinal contents.

In conclusion, tamoxifen is extensively metabolized. Small amounts of hydroxylated metabolites are detected in serum, where tamoxifen and the most abundant metabolites are found to be highly protein bound. Both tamoxifen and its serum metabolites are readily distributed into pericardial, pleural and peritoneal effusions, whereas only trace amounts seem to cross the blood-brain barrier. Moderate quantities exceeding the free fractions were detected in saliva. Conjugated, hydroxylated metabolites are the prevailing species in excretory fluids like urine and bile, and formation of these compounds may be important for tamoxifen elimination.

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